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CIÊNCIA ANIMAL- ESPECIALIDADE EM NUTRIÇÃO

Processed animal by-products as sustainable ingredients in diets for European seabass *(Dicentrarchus labrax)*

Inês Gomes Campos

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Tese de Candidatura ao grau de Doutor em Ciências Animal, Especialidade em Nutrição, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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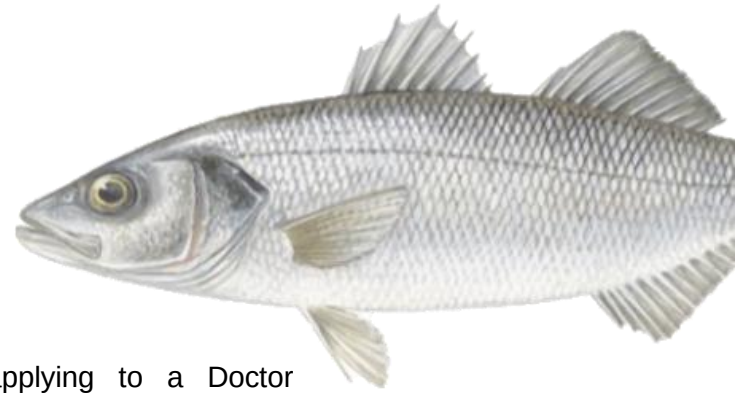

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No cumprimento do disposto no Decreto-Lei nº 204/2018 de 23 de Outubro, declara-se que a autora desta Tese participou na conceção e na execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos.

Nesta tese incluem-se ainda cinco artigos científicos publicados em revistas internacionais resultantes de uma parte dos resultados obtidos no trabalho experimental, referenciados como:

Campos, I., E. Matos, A. Marques and L. M. P. Valente (2017). "Hydrolyzed feather meal as a partial fishmeal replacement in diets for European seabass (*Dicentrarchus labrax*) juveniles." Aquaculture **476**: 152-159.

Campos, I., E. Matos, C. Aragão, M. Pintado and L. M. P. Valente (2018). "Apparent digestibility coefficients of processed agro-food by-products in European seabass (*Dicentrarchus labrax*) juveniles." Aquaculture Nutrition **24**: 1274–1286.

Monteiro, M., E. Matos, R. Ramos, I. Campos and L. M. P. Valente (2018). "A blend of land animal fats can replace up to 75% fish oil without affecting growth and nutrient utilization of European seabass." Aquaculture **487**: 22-31.

Campos, I., E. Matos, M. R. G. Maia, A. Marques and L. M. P. Valente (2019). "Partial and total replacement of fish oil by poultry fat in diets for European seabass (Dicentrarchus labrax) juveniles: Effects on nutrient utilization, growth performance, tissue composition and lipid metabolism." Aquaculture **502**: 107-120.

Campos, I., E. Matos and L. M. Valente (2019). "Ability of European seabass (*Dicentrarchus labrax*) to digest rendered animal fats from fish, poultry and mammals." Aquaculture Nutrition **in press**.

Legal Details

In compliance with what is stated in Decree-Law no. 204/2018 of 23 October 23, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results shown, as well as in their interpretation and the writing of respective manuscripts.

This thesis includes five scientific papers published in international journals from part of the results obtained in the experimental work, referenced to as:

Campos, I., E. Matos, A. Marques and L. M. P. Valente (2017). "Hydrolyzed feather meal as a partial fishmeal replacement in diets for European seabass (*Dicentrarchus labrax*) juveniles." Aquaculture **476**: 152-159.

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Abstract

The current low availability and high price of fishmeal (FM) and fish oil (FO) has led to a need for the reduction of the use of these ingredients in fish feed as protein and lipid sources, respectively. This has led to a search for alternative ingredients that must be economically and environmentally more sustainable, but still assure fish nutrient requirements. The agro-food industry generates large amounts of by-products that, after proper processing, could become valid fishmeal and fish oil substitutes in European seabass (*Dicentrarchus labrax*) diets. Using these locally generated by-products as aquafeed feedstuffs would not only increase the sustainability of this sector by reducing the use of fishmeal and fish oil from fisheries, but also contribute to a functional circular economy by reintroducing such by-products into valued production chains.

In Chapter II, several processed agro-food by-products (wheat germ, okara meal, poultry by-product meal, steam and enzymatically hydrolyzed feather meal, β-lactoglobulin and large peptides (>3kDa) obtained from brewer's yeast and from processing fish by-products) were evaluated as potential alternative protein sources in seabass juveniles. This was done by assessing their chemical composition and their *in vivo* apparent digestibility coefficients (ADCs) in juveniles. Protein ADC's were above 84% for steam hydrolyzed feather meal, enzymatically hydrolyzed feather meal, poultry by-product meal, wheat germ, β-lactoglobulin and the peptides from fish by-products. These ingredients also had high essential amino acids' ADC values (84-98%), while okara and the yeast peptides were poorly digested (73-76%).

Considering the digestibility results, market availability and price of the analyzed feedstuffs, the steam hydrolyzed feather meal (HF) was selected as the best candidate to be used as fishmeal replacement for juvenile seabass. The effects of replacing 28, 55 and 76% fishmeal with HF were evaluated in an 18 weeks growth trial (Chapter III). The replacement of 76% fishmeal by HF did not impair growth performance, nutrient gain, muscle fatty acid composition and humoral non-specific immune parameters of seabass juveniles. Furthermore, the dietary inclusion of HF improved phosphorus ADC, significantly decreasing P emissions into the environment.

The second stage of this thesis evaluated rendered animal fats as potential candidates to replace fish oil in seabass diets. To do that, three rendered animal fats were selected to assess the *in vivo* apparent digestibility coefficients of macronutrients and fatty acids in seabass juveniles in Chapter IV. FO from rendering fish by-products (skin, meat and viscera), poultry fat (PF) and mammal fat (MF, a mixture of about 70% lard and 30% beef tallow) from rendering either poultry or mammal by-products (viscera, skin, bruised meat and bones) were

added at 14%, as supplemental fats, to a practical feed formulation blend commonly used by the feed industry. Both PF and MF had good overall digestibility results, and therefore they were both tested as fish oil replacements in two separate growth trials: one using PF as single fish oil replacement at 25, 50, 75 and 100% (Chapter V), and the other one using a PF and MF mixture (50:50, PFMF) as 50, 75 and 100% fish oil replacement (Chapter VI).

Chapter V and Chapter VI evaluated the effects of replacing fish oil by either PF or PFMF on the nutrient utilization, growth performance, tissue composition and lipid metabolism in seabass juveniles. The use of soy lecithin as an emulsifier agent was also evaluated when PF totally replaced fish oil (Chapter V). Fish oil was completely replaced by PF without impairing feed intake, growth performance and nutrient utilization, but this was only possible up to 75% when the mixture of PF and MF was used. The fatty acid profiles of the tissues analyzed generally reflected those of the diets. Moreover, despite the alterations in the muscle fatty acid profiles, seabass fed diets with up to 75% fish oil replacement (by either PF or PFMF) still provided the recommended EPA and DHA levels for human consumption.

Finally, on Chapter VII, a Life Cycle Assessment (LCA) study was implemented to evaluate the environmental impacts of rendering poultry by-products into HF, PF and poultry by-product meal (PBM, a co-product of PF that can also be used as a FM replacement). The results obtained in this chapter show that the life cycles of HF and PBM generate less impacts than the production of fishmeal in all the categories analyzed. Moreover, the life cycle of PF generates less impacts than the life cycle of fish oil. These results indicate that using such land animal sources as FM or FO replacements would substantially decrease the environmental impacts associated with aquafeeds. Furthermore, the LCA study allowed the analysis of some scenarios regarding the use of different fuels in the rendering plants, which enabled the suggestion of some alternatives that could help decrease their environmental impacts.

This thesis was performed in an industrial setting, under the partnership of the company Soja de Portugal, and focused on helping increase the sustainability of the aquafeed sector. The combination of the results obtained in the different chapters provides valuable information that enables the feed industry to make more conscientious decisions regarding feed formulations and assure a more sustainable development of the sector.

Resumo

A baixa disponibilidade e o elevado preço atuais da farinha (FM) e do óleo de peixe exigem a redução destes ingredientes como fontes proteicas e lipídicas, respetivamente, em ração para peixes. Isto levou à procura de alternativas que sejam não só mais sustentáveis a nível económico e ambiental, mas também que se adaptem às necessidades nutricionais dos peixes. A indústria agroalimentar gera uma enorme quantidade de subprodutos que, após processamento adequado, podem tornar-se válidos substitutos para a farinha e o óleo de peixe em dietas para robalo (*Dicentrarchus labrax*). Usar estes subprodutos, gerados localmente, como ingredientes em dietas para peixe pode não só aumentar a sustentabilidade deste setor (pela redução do uso de farinha e óleo de peixe), mas também contribuir para uma economia circular, através da reintrodução destes ingredientes na economia local.

No Capítulo II desta tese, vários subprodutos agroalimentares (gérmen de trigo, okara, farinha de aves, farinha de penas (hidrolisada a vapor), farinha de penas (hidrolisada enzimaticamente), β-lactoglobulina e péptidos grandes (>3kDa) resultantes da hidrólise de levedura de cerveja e de subprodutos de peixe, da indústria conserveira) foram avaliados como potenciais fontes proteicas em robalos juvenis. Isto foi feito através da análise da composição química destes ingredientes e da determinação da digestibilidade *in vivo* dos seus nutrientes. A digestibilidade da proteína das farinhas de penas, da farinha de aves, do gérmen de trigo, dos péptidos dos subprodutos de peixe e da β-lactoglobulina foram superiores a 84%. Estes ingredientes tiveram também uma digestibilidade elevada dos aminoácidos essenciais (84-98%), que foi mais baixa para a okara e os péptidos de levedura (73-76%).

Considerando a digestibilidade, disponibilidade e preço de mercado dos vários ingredientes, foi selecionado um deles (farinha de penas hidrolisada a vapor, HF) para substituir farinha de peixe em robalos juvenis. Os efeitos da substituição de 28, 55 e 76% de farinha de peixe por HF foram avaliados num ensaio de crescimento de 18 semanas (Capítulo III). A substituição de farinha de peixe até 76% não afetou o crescimento, ganho de nutrientes, perfil de ácidos gordos do músculo ou os parâmetros imunes humorais dos juvenis de robalo. Além disto, a inclusão de HF melhorou a digestibilidade de fósforo das dietas, diminuindo significativamente as emissões deste composto para o ambiente.

A segunda fase desta tese serviu para avaliar gorduras animais como potenciais substitutos do óleo de peixe em dietas de robalo. Para este efeito, três gorduras obtidas através do processamento de subprodutos animais foram selecionadas e a digestibilidade *in vivo* dos seus macronutrientes e ácidos gordos foi avaliada no Capítulo IV. Óleo de peixe, obtido a partir de subprodutos de peixe (pele, carne e vísceras), gordura de aves (PF) e gordura de mamíferos (MF, uma mistura de cerca de 70% banha de porco e 30% gordura de

vaca), obtidas a partir do processamento de subprodutos de aves ou de mamíferos, respetivamente (vísceras, pele, carne pisada e ossos), foram adicionadas em 14% numa formulação tipicamente utilizada na indústria de rações para robalo. Tanto a PF como a MF tiveram boa digestibilidade no geral e, como tal, foram ambas selecionadas como substitutos de óleo de peixe em dois ensaios de crescimento diferentes: um utilizou PF como substituto direto de óleo de peixe a 25, 50, 75 e 100% (Capítulo V), e o outro utilizou uma mistura de PF e MF (50:50, PFMF) para substituir 50,75 e 100% do óleo de peixe (Capítulo VI).

No Capítulo V e no Capítulo VI foram avaliados os efeitos da substituição do óleo de peixe por PF ou por PFMF na utilização de nutrientes, performance zootécnica, composição de tecidos e metabolismo lipídico de robalos juvenis. O uso de lecitina de soja como agente emulsificante foi testado no ensaio que utilizou PF como substituto direto do óleo de peixe (Capítulo V). A substituição total do óleo de peixe por PF foi possível sem afetar o consumo de alimento, crescimento e utilização dos nutrientes, mas isto foi possível apenas até 75% com PFMF (37.5% PF + 37.5% MF). De um modo geral, os perfis de ácidos gordos dos tecidos analisados refletiram os das dietas. Contudo, apesar das alterações nos perfis de ácidos gordos do músculo, os robalos alimentados com as dietas com até 75% de substituição de óleo de peixe (com PF ou PFMF) ainda foram capazes de fornecer os níveis de EPA e DHA recomendados para consumo humano.

Finalmente, no Capítulo VII, foi realizada uma Análise de Ciclo de Vida (LCA) para avaliar os impactos ambientais do processamento dos subprodutos de aves para produzir HF, PF e farinha de aves (PBM, um coproduto de PF que pode substituir farinha de peixe). Os resultados obtidos neste capítulo mostram que os ciclos de vida da HF e da PBM geram menos impactos ambientais do que a produção de farinha de peixe em todas as categorias analisadas. Além disto, o ciclo de vida da PF gera menos impactos do que o ciclo de vida do óleo de peixe. Estes resultados indicam que utilizar estas fontes de animais terrestres como substitutos da farinha de peixe ou do óleo de peixe pode reduzir substancialmente os impactos ambientais associados com as rações de peixe. Além disto, o estudo de LCA possibilitou a análise de alguns cenários relativamente à utilização de diferentes combustíveis nas unidades de processamento dos subprodutos, o que permitiu a sugestão de algumas alternativas que permitiriam diminuir os impactos ambientais destas unidades.

Esta tese foi executada em ambiente empresarial, com a parceria da empresa Soja de Portugal, e foi focada em encontrar estratégias para aumentar a sustentabilidade do setor de produção de rações para peixes. Os resultados obtidos nos diferentes capítulos desta tese fornecem informação valiosa que permitirão à indústria de ração para peixes a seleção mais consciente relativamente a formulações alimentares e assegurar um desenvolvimento mais sustentável deste setor.

Chapter I

General introduction

Aquaculture and nutrition

The evolution of fisheries and aquaculture

The ever-growing world population, allied with climate change scenarios, competition for natural resources, and economic uncertainty, has resulted in one of the world's biggest challenge – providing food to over 9 billion people by 2050 (FAO, 2016). With this in mind, the 2030 Agenda for Sustainable Development, adopted by the UN member states in September 2015, has set 17 sustainable development goals, including approaches on fisheries and aquaculture (goal 14: Conserve and sustainably use the oceans, seas and marine resources for sustainable development), to ensure sustainable development in economic, social and environmental terms (Assembly, 2015).

While capture fisheries have been relatively static since around 1990, aquaculture production has grown outstandingly (Figure 1), providing 47% of the total aquatic production (or 53% if indirect food uses are excluded) in 2016, being able to provide an increased fish supply for human consumption (FAO, 2018b). However, great efforts are necessary in order to meet the continuous demand for fish supply according to the 2030 Agenda: even though global supply of fish for human consumption has outpaced population growth between 1961 and 2016 (3.2 to 1.6%, respectively), global *per capita* fish consumption is also increasing (from 9 kg in 1961 to 20.2 kg in 2015) and is estimated to keep growing (FAO, 2018b). In Europe, Portugal has the third highest *per capita* fish consumption, with about 55.9 kg reported in 2015, with the main consumed products being salted and dried cod, canned sardines and tuna, horse mackerel, gilthead sea bream, salmon and hake (EUMOFA, 2018b). In 2015, fish consumption was responsible for nearly 20% of the animal protein intake of more than 3.2 billion people (FAO, 2018b). This global increase in fish consumption brings beneficial effects to people's health, since fish is not only a source of essential amino acids, minerals and vitamins, but also a great source of essential fatty acids, in particular the omega-3, which are deeply associated with cardiovascular protection and nervous system development.

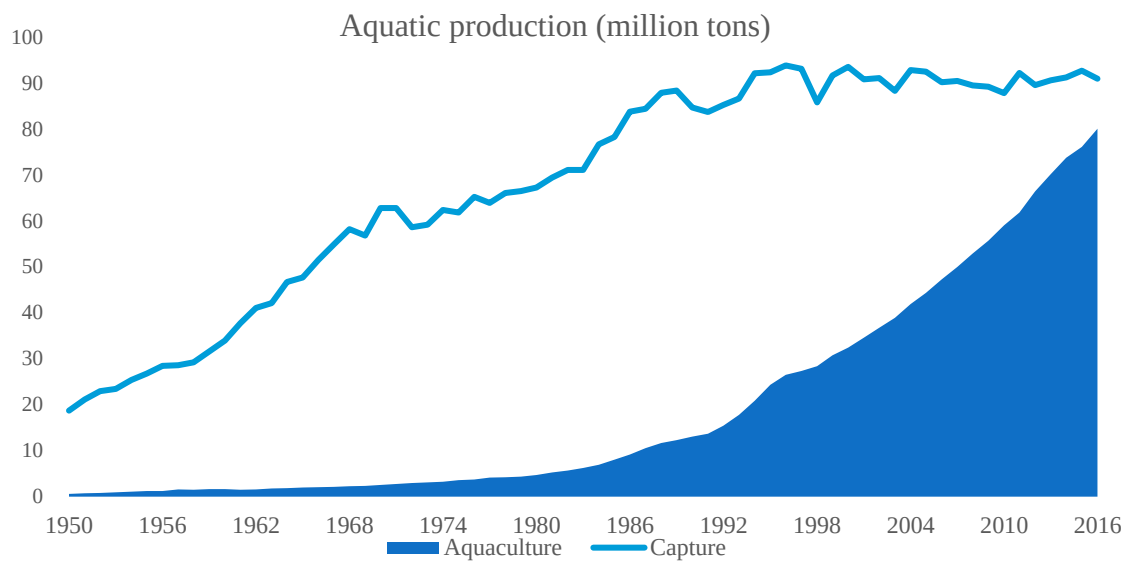

Figure 1. World aquatic production (in million tons), wild capture *vs* aquaculture production (excluding aquatic plants and mammals). Data collected from FAO (2018a), FishStatJ, version 3.04.5.

Despite remarkable progress in some areas, according to FAO, the overall state of world's marine fish stocks keeps declining. Fish stocks being harvested at biologically sustainable levels have continuously decreased from 90 to 67% between 1974 and 2015. Of those 67%, 60% corresponded to stocks at the threshold of sustainability, while only 7% corresponded to underfished stocks. However, even if underfished stocks have continuously decreased since 1974, fishery stocks on the verge of sustainability have increased after 1989, probably due to the implementation of management policies (FAO, 2018b). The portion of total produced fish (farmed and fished) used in direct human consumption over the last decades has increased from 67 to 88% (corresponding to more than 151 million tons) between the 1960's and 2016 (FAO, 2018b).

Since there is no prediction for fishery stocks' recovery from overfishing or at least from being fished at the verge of sustainability, we must rely on aquaculture to supply the necessary fish production to accompany the demands of an ever-growing population. Nonetheless, the growth of aquaculture brings other challenges, some of them common to the fisheries industry, like climate change and diseases, and the competition for resources with other industries, namely feedstuffs, that will be further discussed in the chapter dedicated to fishmeal and fish oil replacement in aquafeeds (Fishmeal and fish oil replacement in aquafeeds).

European seabass

European seabass (*Dicentrarchus labrax*) is a marine fish species (Figure 2) that is both eurythermal (5 to 28 °C) and euryhaline (3 to 35‰) and therefore, in the wild, can be found in the littoral zone, in estuaries, brackish-water lagoons and occasionally in freshwater rivers (FAO, 2017a). In the wild, they are known to go upstream in the summer and to migrate offshore in the colder weather, and while young fish form schools, adult individuals are less gregarious. Adults can achieve over 6 kg body weight and 70 cm in length, and they attain sexual maturity at about 30-40 or 36-46 cm (for males and females, respectively). In the wild, a single breeding season occurs each year during winter or early spring (for Mediterranean and Atlantic populations, respectively). Seabass are predators and in the wild their feeding range includes small fish, prawns, crabs and cuttlefish.

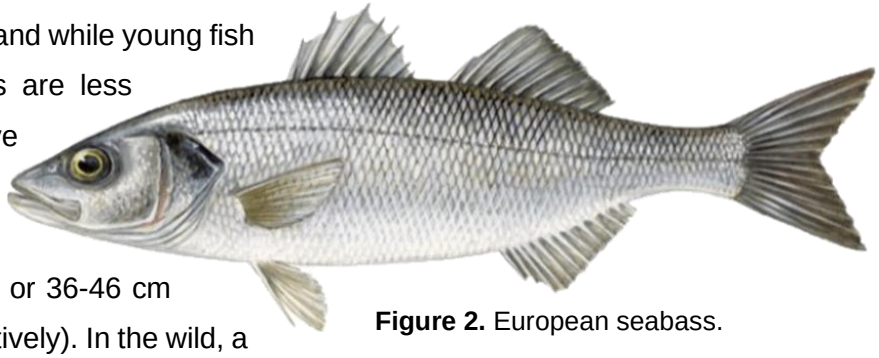

Figure 2. European seabass.

Seabass production was initially extensive and associated with salt production areas (salt was harvested during summer and autumn and fish were cultured during winter and spring) and the cultivated fish were obtained from trapping schools of seabass that lived in those estuarine areas (Figure 3). However, the need for an increased seabass production led to the intensification of this practice.

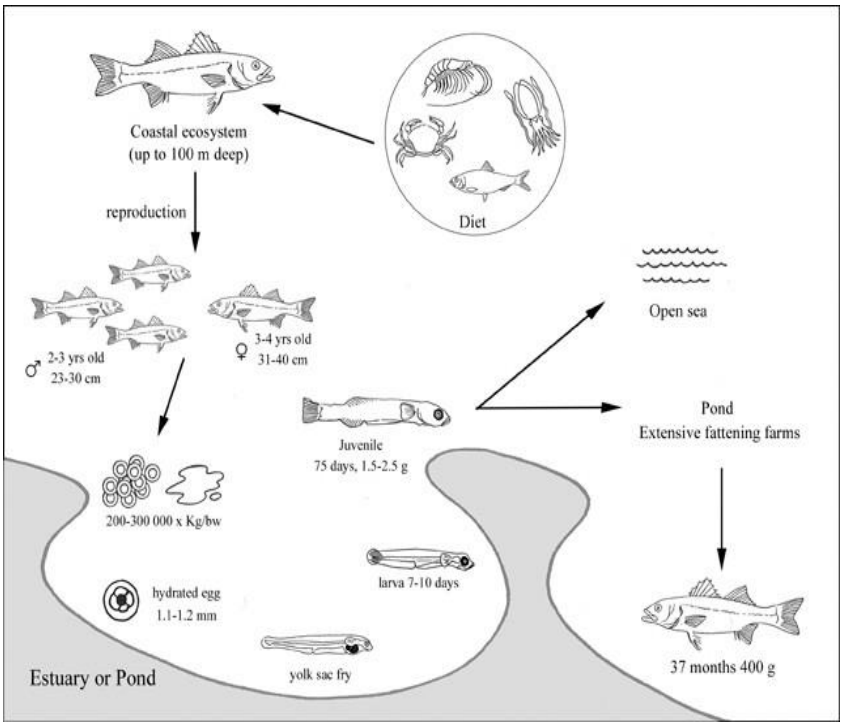

Figure 3. Seabass extensive production cycle (FAO, 2017a).

Economic importance of the species

European seabass is currently one of the most important marine fish species produced in Europe and in the Mediterranean, being one of the 7 most produced aquatic species in the EU (European Commission, 2014a). Its market price is highly related not only to market demands, but also production costs, which in turn are deeply associated with the price of aqua feeds (comprising about 50% of the total costs of aquaculture production; Rana et al. (2009)). Thus, any fluctuation in the price of the feedstuffs has an important economic impact in the final feed price and consequently in seabass production costs.

Even though seabass can be farmed in salt or brackish water ponds or lagoons, it is mainly produced in sea cage farms (Figure 4), which can be anchored close to land, in the open sea, or even inside protective bays. Usually juveniles are obtained from specific hatcheries and are sold to farmers at 1.5 to 2.5 g, being able to reach 400 – 450 g in about 18 to 24 months, depending on water temperature and feeding protocols.

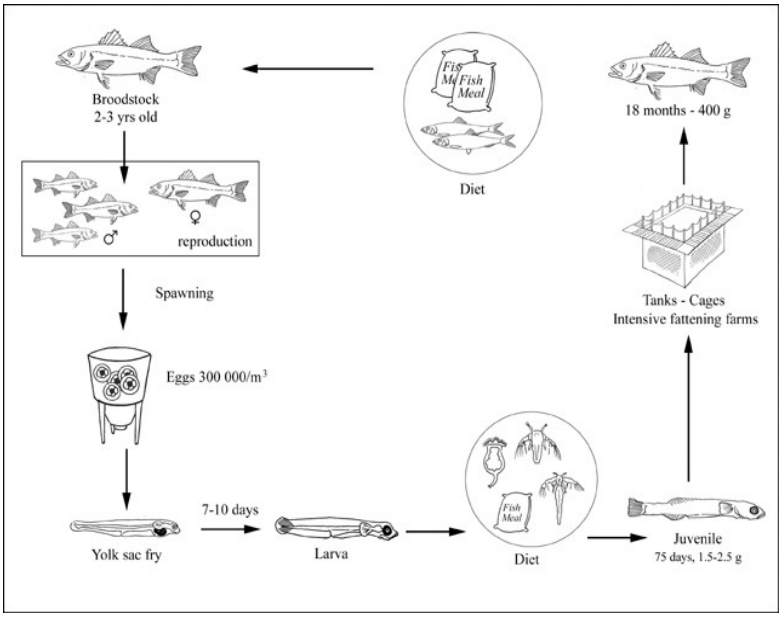

Figure 4. Seabass intensive production scheme (FAO, 2017a).

Seabass aquaculture production started in Italy (1973), followed by Portugal and Greece (1979), but it was only when wild seabass capture reached record values (nearly 8 thousand tons), that aquaculture production rose above one thousand tons to keep up with the market demand for this species1987. Seabass aquaculture production kept growing at an outstanding rate, surpassing wild capture in 1991 and currently accounting for over 30 times the capture supply (Figure 5). Currently, the main producer of European seabass is Turkey, followed by Greece, Spain and Egypt, and total aquaculture production adds up to almost two hundred thousand tones at the moment (Figure 6). While Portugal was one of the first countries with seabass aquaculture production, once accounting for 10% of its total (with only 30 tons), the country's production has peaked in 2006 with 1600 tons and in 2016 was down to 400 tons.

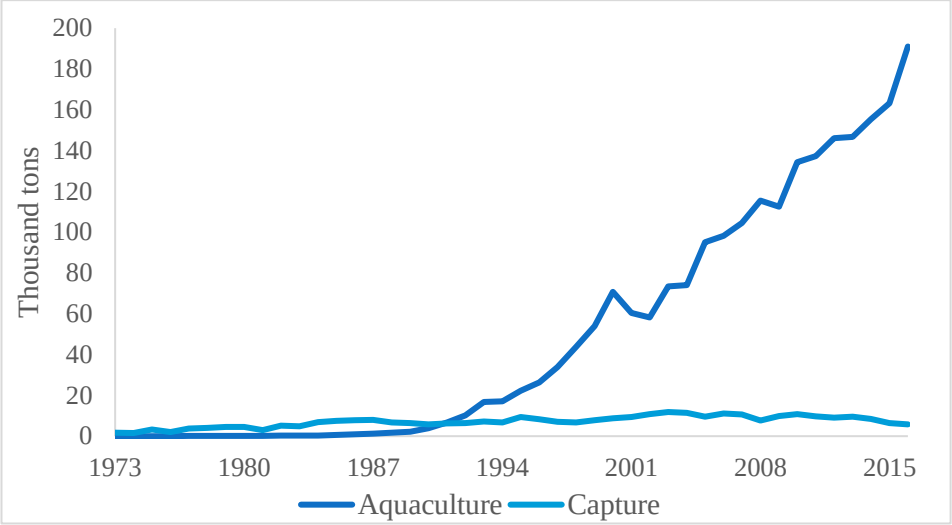

Figure 5. European seabass production, in tons, either from aquaculture or wild caught. Data collected from FAO (2018a), FishStatJ, version 3.04.5.

According to the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA, 2018b), most of the seabass trade is performed within the European Union. In 2016, the intra-EU export market was dominated by Greece (62%), followed by Spain (13%), Croatia (7%) and Italy (4%), while the import market was dominated by Italy (41%), followed by Spain (12%), Portugal (11%) and France (11%).

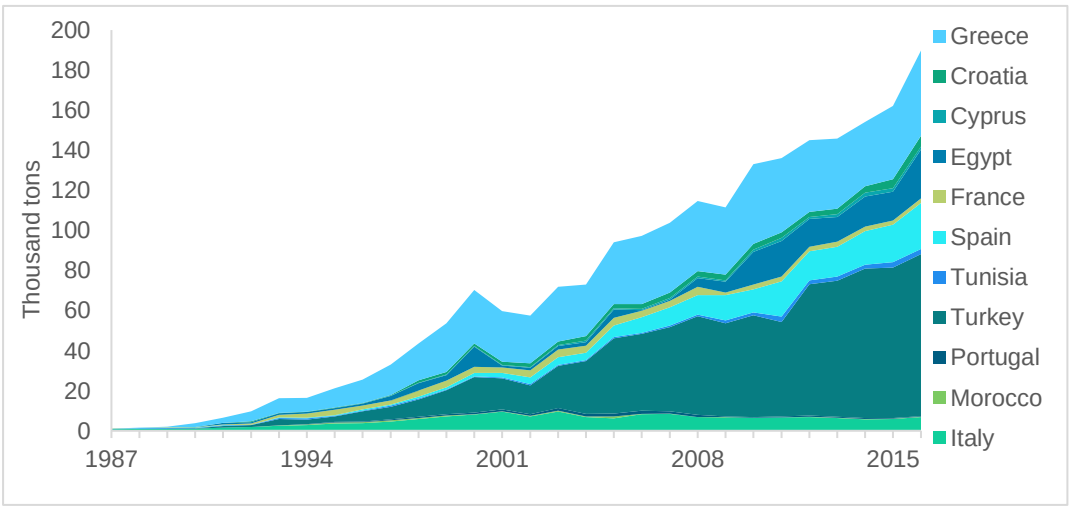

Figure 6. European seabass main aquaculture producers. Data collected from FAO (2018a), FishStatJ, version 3.04.5.

In absolute terms, the EU members leading the seabass market are Italy and Spain, representing more than 60% of the total EU market. On the other hand, while the average EU annual *per capita* consumption of this species is 190 g, Portugal is far ahead with an annual *per capita* consumption of 680 g, followed by Cyprus, Italy and Spain, which all rank above 500 g (EUMOFA, 2018b).

Nutritional requirements

Although European seabass is very important in Mediterranean aquaculture, studies quantifying nutrient requirements of this species are not abundant. Reflecting its carnivore nature, seabass has a very high protein requirement. Early studies estimated an extremely high protein requirement for seabass juveniles (52-60% of the diet, Metailler et al. (1981)), but further methodologies have been refined and reduced the estimated juveniles' requirements to about 43 to 48% dietary protein, provided that proper dietary digestible energy is supplied (Dias et al., 1998; Peres & Oliva-Teles, 1999c). Seabass protein requirement is different depending on whether weight gain or protein gain are used as criteria, but seems to be unaffected by rearing temperature (Hidalgo & Alliot, 1988; Peres & Oliva-Teles, 1999b).

An increase in fish size has been shown to decrease the required dietary digestible protein (DP) to digestible energy (DE) ratio from 25 to 20 mg DP / kJ DE (for seabass with 50 g or 300 g, respectively), which indicates that for the same amount of DE, larger fish will need less dietary protein (Lupatsch, 2005). Information regarding energy and protein requirements for seabass growth have been used to create models that can help researchers or aquaculture technicians predict overall requirements for a specific growth and body composition (Lupatsch, 2005). In that sense, requirements for energy and protein should be calculated as follows:

$$\text{Energy requirement } (kJ/fish/day) = 45.4 \times \text{BW (kg)}^{0.8} + 1.44 \times \text{energy gain}$$

$$\text{Protein requirement } (g/fish/day) = 0.61 \times \text{BW (kg)}^{0.7} + 1.92 \times \text{protein gain}$$

Additionally, using data on seabass growth and considering the fish body weight (BW), energy and protein whole-body content and the rearing temperature conditions, equations that were best adjusted to those data allowed the formulation of the following models:

$$\text{Weight gain (g)} = 0.0196 \times \text{BW (g)}^{0.517} \times \text{e}^{0.065 \times \text{Temp}}$$

$$\text{Energy content of fish (kJ/g)} = 5.17 \times \text{BW(g)}^{0.107}$$

$$\text{Protein content of fish (\%)} = \frac{17.1 \text{ g}}{100 \text{ g BW}}$$

Although crude protein requirements have been extensively studied, fish do not require absolute proteins, but rather the amino acids that compose them. Fish can't synthesize all amino acids, so they need to obtain some (essential amino acids, EAA) dietary supply. Seabass requires the same ten EAA as other finfish: arginine (Arg), lysine (Lys), histidine (His), threonine (Thr), isoleucine (Ile), leucine (Leu), valine (Val), methionine (Met), phenylalanine (Phe) and tryptophan (Trp). Some studies using dose-response curves have been able to quantify individual requirements of Arg, Lys, Met, Trp and Thr (Thebault et al., 1985; Tibaldi & Lanari, 1991; Tibaldi et al., 1993a, 1993b; Tibaldi & Tulli, 1999), while Kaushik (1998) estimated the requirements of other EAA based on the whole-body amino acid content.

Table 1. Whole-body amino acid composition and amino acid requirements in seabass (% protein).

Amino acid	Whole-body composition (1)	Estimated requirement	Reference
Arg	7.5 – 8.4	4.1 – 4.6	1, 2
Lys	7.6 – 7.9	4.8	1, 3
His	2.4 – 2.7	1.6	1
Ile	4.1 – 4.3	2.6	1
Leu	7.1 – 7.2	4.3	1
Val	4.6 – 4.7	2.9	1
Met + Cystine	3.6 – 3.7	2.3 – 4.4	1, 4
Phe + Tyrosine	8.2 – 8.4	2.6	1
Thr	4.3 – 4.4	2.6 – 2.7	1, 5
Trp		0.5 – 0.6	1, 6

1: Seabass whole-body amino acid composition, the range corresponds to values obtained for 3.3 and 233 g fish (Kaushik, 1998); 2: Tibaldi et al. (1993a); 3: Tibaldi and Lanari (1991); 4: Thebault et al. (1985); 5: Tibaldi and Tulli (1999); 6: Tibaldi et al. (1993b).

There is no required amount of fat *per se* in seabass diets, but a 18-20% dietary fat level is recommended for maximum protein utilization efficiency. Fat has a protein sparing effect in seabass, increasing protein utilization efficiency up to 18-20% dietary fat levels, but higher levels (30%) impaired its growth (Peres & Oliva-Teles, 1999). Regarding fatty acids, however, long chain *n*-3 highly unsaturated fatty acids (LC-HUFA) like EPA and DHA are essential for this species, as it cannot synthetize them. Early studies recommend an LC-HUFA dietary level of 1% dry diet for seabass larvae (Coutteau et al., 1996), while recent studies suggest that juvenile seabass require at least 0.7% *n*-3 LC-HUFA of the dry diet, with a DHA/EPA ratio of 1.5:1 to achieve a good growth performance (Skalli & Robin, 2004).

Dietary carbohydrates also have a protein sparing effect in seabass. However, since it is a carnivorous species, high dietary levels of carbohydrates (> 30% dietary starch) can impair growth (Alliot et al., 1979), even when they are presented in the highest digestible form (pre-treatment is necessary on vegetable feedstuffs to reduce the complexity of the carbohydrates and enhance their digestibility in seabass). Regarding mineral and vitamin requirements for European seabass, data are very scarce. Water soluble vitamin requirements have been suggested to be similar to those reported for salmonids (Kaushik et al., 1998). Fournier et al. (2000) reported a minimum of 5 mg ascorbic acid per kg of seabass feed for maximal growth and normal skin collagen concentration, but the same study suggests higher levels could be required. No data is available on fat-soluble vitamins requirements, but a dietary level above 100 mg vitamin E per kg of feed has been recommended to prevent lipid peroxidation and increase fillet shelf life (Gatta et al., 2000; Pirini et al., 2000). Concerning minerals, only phosphorus dietary requirement (0.65%) has been determined in seabass (Oliva-Teles & Pimentel-Rodrigues, 2004). While the bioavailability of phosphorus is higher in animal than in vegetable meals, for being present in the latter as phytate, which is not available to monogastric animals, this reduced availability has been shown to improve with the dietary inclusion of microbial phytate (Oliva-Teles et al., 1998).

Protein metabolism in fish

Amino acids can be generated within the body from three different sources: through dietary protein and free amino acids, through synthesis (nonessential amino acids) from other metabolic intermediates, or from the breakdown of tissue proteins (Emery, 2005; Guillaume et al., 2001). Amino acids also have three metabolic destinations: synthesis of body protein (for growth and reproduction or for maintenance, replacing existing proteins), of other nitrogenous compounds, or oxidation to carbon dioxide, water and urea (Figure 7; Wilson (2002)).

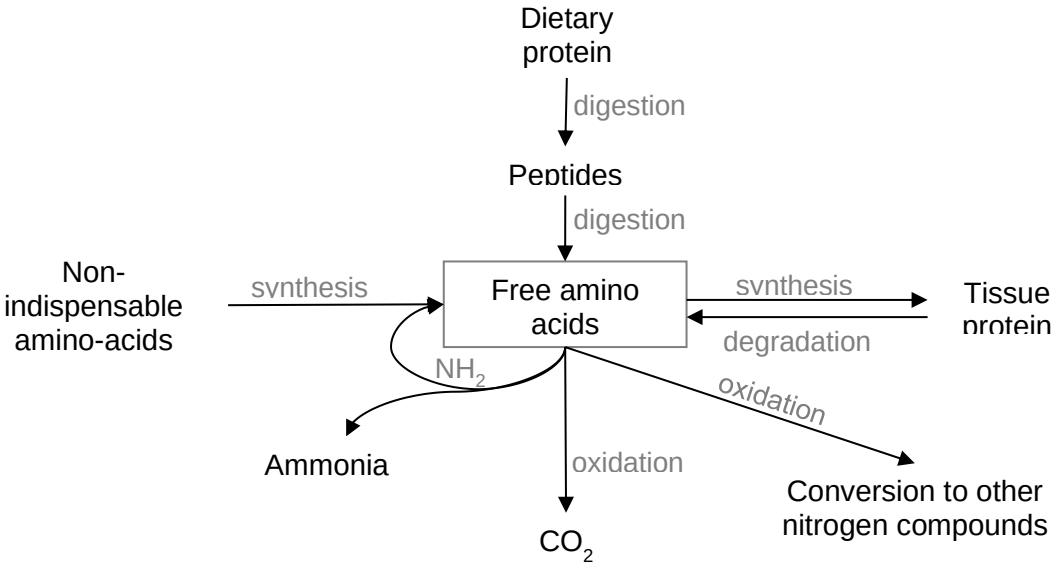

Figure 7. Overview of amino acid metabolism. Adapted from Guillaume et al. (2001).

When protein is ingested, it is digested into small peptides by enzymes secreted into the gastrointestinal tract. These small peptides are absorbed by the intestinal epithelium and hydrolyzed into free amino acids (Wilson, 2002). The amino acids enter the systemic circulation to be transported into different body tissues, where they are metabolized. In fish, studies have shown that about 25 to 55% of the ingested amino acids are deposited as body protein (Mambrini & Guillaume, 2001). Since there is as strong link between protein deposition and weight gain, live weight gain and amino acid requirements are also closely related (NRC, 2011). The catabolism of tissue proteins is an important amino acid source: in fish, most of the proteins synthesized in the liver and gastrointestinal tract are turned over into free amino acids (less than 10% are fixed as tissue protein), while most of the muscle protein synthesized is retained (50-70%; Cowey and Luquet (1983)). Through the process of amino acid turnover, in fish 40-50% of tissue protein is catabolized, corresponding to less than 50% of the free amino acid pool that can be used for syntheses (Cowey & Luquet, 1983). This is much lower than what is observed in mammals (70-80% of the free amino acid pool), indicating that fish growth is more dependent on dietary protein than mammals (Millward et al., 1976). Thus, inadequate

dietary protein supplies lead to growth impairment and weight loss, as protein from less vital tissues have to be hydrolyzed to support the functions of more vital tissues. On the other hand, if dietary protein is in surplus, it will be used to produce energy (Wilson, 2002).

The amino acid catabolism can be divided into three major types: inevitable catabolism occurs even when the amino acid supply is below a species requirement level, compromising protein deposition; preferential catabolism uses amino acids to produce energy whenever energy intake is restricting body protein deposition, which means that essential amino acids are being shifted from protein synthesis to catabolism in order to achieve specific metabolic needs. In fish, separating inevitable from preferential catabolism is not easy, as amino acids seem to provide a substantial fraction of the total energy requirements (NRC, 2011). Finally, when amino acids are in dietary surplus (above the requirement levels for protein deposition, maintenance, inevitable and preferential catabolism), these amino acids will be additionally catabolized. Ergo, if a diet is deficient in some essential amino acids, this will not only impair the protein deposition but also the retention of the remaining amino acids, promoting their catabolism and deamination (NRC, 2011). Furthermore, single amino acid dietary deficiencies can lead to decreased feed intake (de la Higuera, 2001). In seabass this response seems to vary accordingly to the deficient amino acid, with the biggest reduction in voluntary feed intake being observed with tryptophan deficiency (Tibaldi & Kaushik, 2005).

Lipid metabolism in fish

The term *lipid* defines a rather varied group of compounds that share the characteristic of being water-insoluble, but soluble in some organic solvents like alcohols, chloroform, ethyl ether and hydrocarbons (Dupont, 2005). The solubility of these compounds highly depends on their polar or neutral characteristics, which also define the function and structure of lipids in living organisms (Dupont, 2005). Several lipid classes exist according to their composition and structure: triacylglycerols (TAG, composed by 3 fatty acids esterified to a L-glycerol molecule), wax esters (one fatty acid esterified to a fatty alcohol molecule), phosphoglycerides (two fatty acids esterified to an L-glycerol 3-phosphatase molecule), sphingolipids (one fatty acid and a sphingosine molecule) and sterols (including cholesterol, which can be esterified to a fatty acid or exist in non-esterified form in cell plasma membranes; Sargent et al. (2002)).

Apart from cholesterol, which as previously mentioned may exist in the non-esterified form, all the mentioned lipid classes contain fatty acids. Fatty acids (FA) are hydrocarbons, with variable carbon chain length (two or more carbon atoms), and they mostly occur as triacylglycerols (Dupont, 2005). FA nomenclature is based on their chain length, number of double bonds (unsaturation degree) and position of the double bonds. So, for instance, 16:0

is a FA with 16 carbon atoms and with no double bonds, while 18:1*n*-9 and 18:1*n*-7 are FA with an 18 carbon-chain and a single double bond on the 9th and 7th carbon, respectively. Thus, the nomenclature saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) stands for FA that have no double bonds, a single double bond, and two or more double bonds, respectively (Sargent et al., 2002). Regarding the position of the double bonds, the most common series are *n*-3, *n*-6 and *n*-9. In human nutrition, 18:3*n*-3, 20:5*n*-3 (eicosapentaenoic acid, EPA) and 22:6*n*-3 (docosahexaenoic acid, DHA) are linked to cardiovascular health, decreased inflammation and neurological development, being essential for normal growth and health (Sargent, 1997; Siriwardhana et al., 2012).

Lipid digestibility, absorption and transportation

In fish, after being ingested, dietary lipids are usually well digested, except when the melting point of the lipid source is above the fish rearing temperature. This melting temperature is directly related with the saturation degree of the fatty acids that compose the lipid: the higher the degree of unsaturation, the lower the melting point. Hence, lipids richer in PUFA tend to be liquid at room temperature, while SFA-rich fats will be solid (Corraze, 2001; Trushenski & Lochmann, 2009). Also, the melting point of SFA increases with chain length, being higher for 18:0 than for 12:0, decreasing the digestive utilization of the former comparatively to the latter. Lipids are hydrolyzed by several lipases and colipases in the stomach, intestine and cecal lumen, with the pyloric ceca and the anterior intestine being the major sites for this hydrolysis (Rust, 2003). After hydrolysis, FA with 2-10 carbons and glycerol molecules are directly absorbed into the enterocytes, while long-chain FA (longer than 10 carbons) are cleaved by lipase and emulsified by bile salts to create micelles, which are transported into the enterocyte brush border. After the diffusion into the enterocyte, FA are re-esterified into triacylglycerols and phospholipids and, specifically in fish, may form large lipid droplets and remain temporarily stored within the enterocyte (Corraze, 2001).The majority of the re-esterified lipids, however, are combined with proteins to form lipoproteins, more specifically chylomicrons and very low-density lipoproteins (Rust, 2003), which are then transported into the liver through the lymphatic system or via the hepatic portal in some fish species (Corraze, 2001).

In the liver, these fatty acids and others synthesized *de novo* in this organ, are re-esterified into new lipoproteins (very low-density lipoproteins, low-density lipoproteins and high-density lipoproteins) that mediate FA transportation into the peripheral tissues (Sheridan, 1988). When dietary FA are transported to peripheral tissues by lipoproteins, endothelial lipoprotein lipase (LPL) breaks the bonds and unesterified FA can be taken up by the tissue cells (Turchini et al., 2009b) where they are used to produce energy or stored (Corraze, 2001).

Lipid catabolism

Fatty acids play a vital role in all organisms, being used to produce ATP as an energy source through mitochondrial β-oxidation (Sargent et al., 2002). The relative importance of dietary lipids for energy production, compared with protein, is species specific and can be dependent on dietary lipid level, having a protein-sparing effect. The most important tissues for β-oxidation in fish are red muscle, liver and heart. Although white muscle has a low β-oxidation capacity, it comprises about 60% of the fish body and consequently is the major β-oxidation site (Turchini et al., 2009a). β-oxidation studies in fish have suggested a substrate preference for SFA and MUFA over PUFA (Henderson, 1996; Kiessling & Kiessling, 1993). Studies with Atlantic salmon (*Salmo salar*) showed that C18 PUFA (18:2*n*-3 and 18:2*n*-6) and *n*-3 HUFA (including EPA and DHA) are β-oxidized when in dietary surplus, but preferentially retained when in lower dietary levels (Bell et al., 2003; Stubhaug et al., 2007). suggesting that fish are able to select the FA used as substrate for β-oxidation and those that are selectively stored. Moreover, other authors have also shown that dietary *n*-3 HUFA can increase fatty acid uptake to the mitochondria, stimulating total β-oxidation (Kjaer et al., 2008).

Lipogenesis

There is no mechanism for the long-term storage of protein or carbohydrates in animals, in which TAG are the main form of energy storage. Therefore, dietary carbohydrate and protein surplus are converted into fat before storage in adipocytes, through the *de novo* FA synthesis (or lipogenesis) process, which occurs through the FA synthetase (FAS) complex (Corraze, 2001). This enzymatic process is similar to that occurring in mammals, but in fish it occurs mainly in the liver and occasionally in the adipose tissue, while in mammals it mainly occurs in the latter (Henderson & Sargent, 1985). In fish, the reduced FAS activity in the adipose tissue compared to its higher hepatic activity indicates a preference for the liver as lipogenesis site and for the adipose tissue as FA storage (Henderson & Sargent, 1985; Lin et al., 1977a, b).

In the lipogenesis process, NADPH is necessary for reduction power, and it can be provided by three different enzymatic processes (glucose-6-phosphate dehydrogenase, G6PD; malic enzyme, ME; and $NADP^+$-dependent isocitrate dehydrogenase, NADP-IDH). The relative activity of these enzymes varies not only according to species but also to the animal's nutritional and hormonal state (Barroso et al., 1994, 1998, 1999, 2001; Garcia-Jimenez et al., 1993; Kletzien et al., 1994; Sánchez-Muros et al., 1996). However, regardless of their nutritional and hormonal state, some fish species have been shown to obtain NADPH mainly through G6PD, as is the case for European seabass that shows a high activity for this enzyme (Enes et al., 2006a, b) compared to a much lower ME activity in the same species (Alvarez et al., 1998; Dias et al., 2005a; Richard et al., 2006). The dietary composition can also have

impacts on lipogenesis: in Atlantic salmon, high dietary lipid levels decreased the lipogenic activity (Arnesen et al., 1993) and in rainbow trout (*Oncorhynchus mykiss*) and rat hepatocytes the presence of dietary PUFA is reported to inhibit lipogenesis (Alvarez et al., 2000; Zampelas et al., 1995). In addition to being used in the lipogenic pathways, the NADPH produced by lipogenic enzymes can also be used to maintain the cell red-ox state. Since dietary PUFA can increase the requirements for anti-oxidants such as NADPH (Benzie, 1996), its presence can reduce the NADPH available for lipogenesis, potentially decreasing its intensity. The main FA synthesized *de novo* are the SFA myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0), but their relative synthesis proportion varies according to specie (Corraze, 2001). The newly synthesized FA (as well as dietary FA) can be converted to some degree into more unsaturated or FA with longer chains. However, some FA cannot be synthesized by the fish and must be supplied in the food.

Fatty acid requirements

Fish, like other vertebrates, cannot synthesize linoleic (18:2*n*-6) nor alpha-linolenic acid (18:3*n*-3), therefore these FA must be provided in the feed. However, to a certain degree, nearly every fish can convert 18:2*n*-6 and 18:3*n*-3 into *n*-6 and *n*-3 HUFA by successively desaturating and elongating the fatty acid (portrayed in Figure 8).

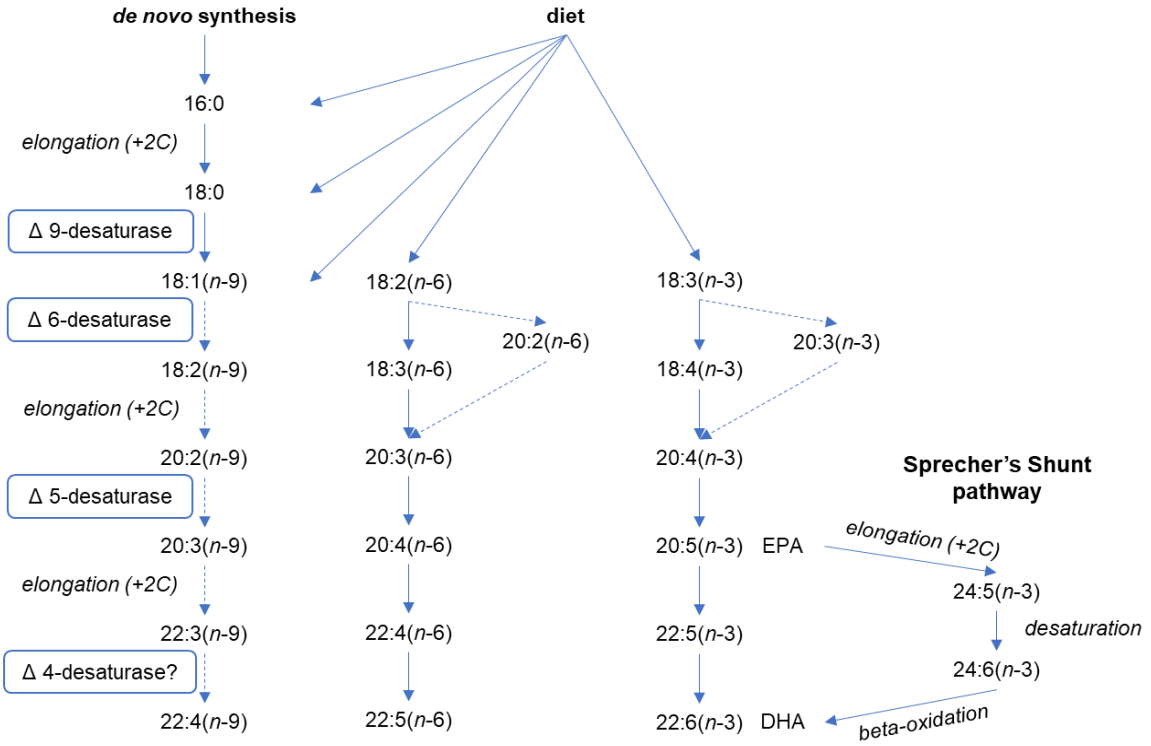

Figure 8. Bioconversion of fatty acids: elongation and desaturation of *n*-9, *n*-6 and *n*-3 PUFA. Adapted from Bell et al. (1986) with the addition of the Sprecher's shunt pathway (Qiu, 2003); -----: minor conversion routes.

Through these elongation and desaturation pathways, most fish can produce *n*-3 and *n*-6 LC-HUFA from dietary PUFA. However, even though marine species like seabass express elongase and Δ5 and Δ6-desaturase activity, the extent of fatty acid conversion is minimal. Therefore, LC-HUFA like EPA and DHA are essential for this species and need to be supplemented in the diet (Turchini & Francis, 2009). Essential fatty acids have a major role as components of phospholipids and therefore as cell membrane constituents. They also serve as substrate for molecules like prostaglandins, leukotrienes and thromboxanes, which have functions on important systems like the nervous or circulatory systems (Corraze, 2001). For this reason, a deficient dietary supply of essential fatty acids can lead to several issues, such as reduced growth performance and a reduced nutritional quality of fish fillet (reduced *n*-3 HUFA).

Tissue lipid deposition

In fish, unlike mammals where fat is only stored in adipose tissue, lipids can be stored in several different tissues, such as muscle, liver, perivisceral adipose tissue and subcutaneous tissue (Corraze, 2001). The digestibility and consequent transport, uptake, elongation, desaturation and β-oxidation processes of fatty acids affect the lipid composition of tissues (stored mainly as TAG and waxes) and cell membranes (as phospholipids). The membrane phospholipids have a preponderance of *n*-3 series FA, which helps the maintenance of membrane fluidity and permeability, particularly at low temperatures (Sargent et al., 2002), due the already mentioned melting point differences among FA (Corraze, 2001). Therefore, *n*-3 FA deficiencies could lead to alterations in membrane phospholipid composition and consequently impact the membrane fluidity. The dietary ratio of neutral to polar lipids can also have effects on the fatty acid composition of a determined tissue. For instance, the tissue's polar lipids fraction has been shown to be less susceptible to reflect the dietary fatty acids profile than the storage fraction (neutral lipids) of tissue lipids (Brodtkorb et al., 1997), so tissues that are composed mainly by polar lipids, such as the heart, are less likely to reflect the dietary FA profile (Kwetegyeka et al., 2011).

Fishmeal and fish oil replacement in aquafeeds

Fishmeal and fish oil are the main protein and lipid sources, respectively, in feeds for carnivorous fish, due to their high palatability, adequate amino acid profile, for being a great source of essential FA and minerals, and, until recently, for having relatively low price and wide availability (Drew et al., 2007a; Gatlin et al., 2007). However, the fast growth of aquaculture has been accompanied by aquafeed production, leading to a depletion of the fishmeal and fish oil stocks and an increase of these feedstuffs' prices (Figure 9; Benedito-Palos et al. (2007); Kaushik (2004); Turchini et al. (2009a)). According to FAO reports (2008), in 2006 aquaculture used 56% and 87% of the world fishmeal and fish oil production, respectively. The increase of total aquafeed production more than three-folded from 1995 to 2008 (7.6-29.2 million tons), growing at about 11% per year, and is expected to keep growing at that rate, reaching 71 million tons by 2020 (FAO, 2016).To maintain the current growth rate of about 8-10% per year up to 2025 (FAO, 2016), the aquaculture industry needs a feedstuff supply that can accompany that growth. Although this was possible in the early history of aquaculture, nowadays the scenario is no longer the same. Since feedstuffs preferentially used in fish feeds are becoming scarce and may compete with other animal feeds, including direct competition with Human nutrition, it makes them environmentally and economically unsustainable (Rana et al., 2009).

The future availability of fishmeal and fish oil is a major concern, thus, the sustainable growth of aquaculture, both economic and environmental, largely depends on the selection of alternative protein and fatty acid sources. However, the selection of such new ingredients must obey several conditions, like being nutritionally adequate to important species in aquaculture and being available in the market with competitive prices (National Research Council, 2011). The price of aquafeeds comprises about 50% of the total aquaculture production costs and the raw materials account for about 75% of the aquafeed costs (Rana et al., 2009). Ergo, any fluctuation in the feedstuffs' price has an important economic impact in the final feed and fish production costs. Ingredients selected as protein sources should also possess certain nutritional characteristics, like low fiber, starch and particularly non-soluble carbohydrates levels, high protein content with an adequate amino acid profile, high nutrient digestibility, good palatability and absence of anti-nutritional factors (Gatlin et al., 2007). Fish oil is the main source of *n*-3 fatty acids, so its replacement by other sources with limited or lacking such HUFA has multiple effects on fish performance, metabolism and final fillet qualities, being a difficult task. The composition of different lipid sources, namely their FA profiles, is responsible for these effects, and, when considering animal fats, it can be highly dependent on the feeding history of the animal (Trushenski & Lochmann, 2009). While studies seem to show that fish oil replacement does not affect survival or growth performance (provided that basal energy and

FA requirements are met), this alteration can have other effects, namely on the tissue FA composition (Turchini et al., 2009b). Seabass tissue fatty acid composition, as with many other teleosts, mainly reflects the dietary fatty acid profile, which can be challenging when using fish oil replacements. Thus, even when alternative lipid sources provide good growth rates, finishing fish oil-based diets may be necessary to obtain the required marketable fillet fatty acid profile (Trushenski & Lochmann, 2009).

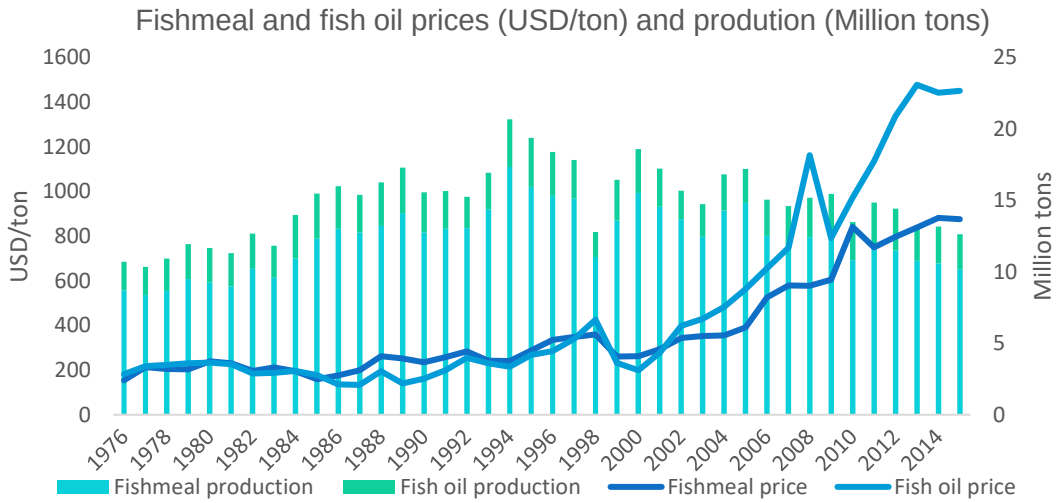

Figure 9. Total world fishmeal and fish oil production (in million tons) and prices (in USD/ton). Data collected from FAO (2017), FishStatJ, version 3.04.5.

Besides all these requirements, the selected feedstuffs should also be environment-friendly in order to reduce the environmental impacts associated with aquaculture production. There is also increasing interest in safe and locally produced protein and fatty acid sources alternative to fishmeal and fish oil, able to reduce the dependency of imported feedstuffs, reducing pressure on fisheries and the carbon footprint of aqua feeds.

Although aquaculture production remains highly dependent on fishmeal and fish oil, the selection and use of alternative protein and lipid sources from vegetable or land animal origin has decreased the use of those feedstuffs. In fact, the total use of fishmeal by the aquaculture sector has decreased from 4.23 to 3.72 million tones between 2005 and 2008 and a decrease down to 3.49 million tones is expected by 2020 (Tacon et al., 2011), mostly due to the decrease in fishing quotas and to the growing use of dietary fishmeal replacers. Contrarily, despite the reduction of fish oil usage in fish feeds, due to the increase in aquafeed production the use of fish oil is still increasing and is expected to slowly rise from 782 000 tones in 2008 to 908 000 tones by 2020 – since there are no cost-effective alternative lipid sources rich in long-chain PUFA, fish oil is still a main fatty acid source for marine finfishes and crustaceans. Nonetheless, fisheries by-products are becoming a great contributor to the available fishmeal and fish oil, as in 2007 one quarter of total fishmeal production already came from by-products and this fraction is expected to keep growing (EUMOFA, 2018; Tacon et al., 2011).

Vegetable alternatives

Most of the research made to identify suitable fishmeal and fish oil replacers in finfish nutrition has been done with ingredients of vegetable origin (Hardy, 2010; Turchini et al., 2009). However, since the use of vegetable meals and oils to replace fishmeal and fish oil has increased substantially, the prices of those ingredients have also suffered a dramatic increase (FAO, 2016). Furthermore, the availability and price of feedstuffs from plant origin can have wide variations, as they are directly influenced by climate changes. Given the current global warming scenario, the dependency of imported feedstuffs like soybean, wheat, rapeseed, maize, rice and palm oil is likely to remain and even increase, since climate change will reduce the probability of self-sufficiency (FAO, 2016). On the other hand, vegetable plants and oils are also commonly used by other markets, including direct human consumption, and therefore using these ingredients in aquafeeds can reduce the availability of these ingredients for human consumption and, inversely, the increase on the use of these ingredients by the human population will lead to an increase in their price (Tacon et al., 2011). Furthermore, since the main suppliers of soybean, wheat and maize are in the American continent and the main consumers are in Europe and Asia, they require transportation, which has associated environmental impacts. We should also consider that fuel and transport price volatility also have a high impact on the price of these feedstuffs and it is unlikely that these ingredients will see a decrease in their current prices (FAO, 2016).

Vegetable ingredients as protein sources for fish

Most plant protein sources have a high carbohydrate content (particularly non-starch polysaccharides), deficient amounts of certain essential amino acids such as lysine and methionine (Gatlin et al., 2007) and a wide variety of antinutritional factors such as protease inhibitors, lectins, saponins and phytic acid, the latter being responsible for reduced availability of phosphorus for fish (Krogdahl et al., 2010). The presence of these antinutritional factors should be controlled when using vegetable ingredients in aquafeeds (Hardy & Barrows, 2002). For instance, the addition of microbial phytase has been shown to significantly improve the phosphorus availability of several vegetable meals for rainbow trout (Cheng & Hardy, 2002b). However, the need to incorporate additives to the feeds to process vegetable ingredients in order to overcome these issues will increase the price of the feed formulation. Advantages and disadvantages of plant feedstuffs commonly used as fishmeal replacement are further detailed in Table 2.

Table 2. Advantages and disadvantages of some plant feedstuffs compared with fishmeal in aquafeeds (adapted from Gatlin et al. (2007)).

Plant substitute	Advantages	Disadvantages
Barley	Well digested protein (87% in hybrid tilapia)	Low crude protein content (9-15%); High in fiber; Low in available phosphorus; Lysine and arginine maybe limiting
Canola meal	Protein content similar to soybean meal (44-48%).	Similar price to soybean meal; low in available phosphorus
Canola protein concentrate	Protein content similar to high-quality fishmeal; Supports growth rates similar to those of fish fed fishmeal-based diets	Amino acid supplements need to overcome limiting amino acid levels; Feeding stimulants are needed to overcome reduced feed intake.
Corn gluten meal	Protein content of 60-73%; highly digestible.	Limited commercial production; Deficient in lysine.
Cottonseed meal	40% crude protein; Can be used up to 30% without growth depression.	Presence of gossypol may have toxic effects.
Peas/lupin	High protein apparent digestibility coefficient	Limiting amounts of lysine and methionine; High levels of carbohydrate (fish cannot metabolize non-starch polysaccharides in lupins); Presence of anti-nutrient quinolizidine alkaloids.
Soybean meal	Economical and nutritious with high crude protein (44-48%); Cystine in higher concentration than in FM.	Levels of tyrosine and the 10 EAA are lower (lysine, methionine, cystine and threonine may be limiting); Crude fat and ash content are lower; High in non-starch polysaccharides; Presence of anti-nutritional factors such as lectin; Low phosphorus availability; Results in: reduced fish feed intake and growth, intestinal enteritis
Soybean protein concentrate; Soy protein isolate	EAA concentration matches or overcomes concentrations in fishmeal.	Methionine and cystine may be limiting; Not economical for large scale use; crude fat and ash content is lower but can be overcome with supplementation.

Potential fishmeal replacers in aquafeeds should be highly digestible ingredients in order to improve fish performance and reduce waste production. However, since the nutrient digestibility of an ingredient varies from species to species due to intestinal physiology differences, results obtained in different species are hard to compare (NRC, 2011).

The nutritional quality of a certain feedstuff depends not only on its source, but also on the applied processing methods, which can affect the bioavailability of its nutrients. To determine their potential as protein sources, the digestibility of some feedstuffs has already been determined for European seabass (Table 3).

Table 3. Apparent digestibility coefficients (ADC) of protein sources in European seabass.

		ADC (%)		
Ingredient	CP (%DM)	Protein	Energy	Reference
Animal ingredients				
Fishmeal, Denmark	73	95	95	1
Fishmeal, Norway	75	96	95	2
LT-94 Fishmeal, Norway	67	94	88	3
Fishmeal, Portugal	70	90	86	2
Fishmeal, Peru	62	94	94	1
CPSP G, France	72	97	95	2
Poultry-meat meal	60 – 62	86 – 97	97	1, 3
Meat meal, defatted	75	92	86	2
Blood meal	97	91	92	2
Spray dried hemoglobin meal	87	91	67	3
Steam hydrolyzed feather meal (HF)	77	67	58	3
Enzymatically HF	82	68	61	3
Vegetable ingredients				
Dry brewer's yeast	48	89	85	1
Soybean meal	46 – 52	89 – 90	82 – 96	1, 2
Soy protein concentrate	65	97	88	1
Rapeseed meal	36	93	78	1
Maize gluten meal	55	91	87	1
Wheat gluten	77	98	93	1
Extruded pea meal	24.5	92.5	84	1
Extruded lupin-seed meal	42.0	93.7	88	1

CPSP – fish soluble protein concentrate;
1 - Tulli and Tibaldi (2001); 2 - Gomes da Silva and Oliva-Teles (1998); 3 - Davies et al. (2009).

Even though data on animal ingredients is available, studies conducted to reduce the use of fishmeal in seabass have been mostly focused on vegetable sources, which is probably a consequence of the animal meals ban in 2001, in the European Union. The logistics and processing of animal products has evolved since, making the data on digestibility outdated. Additionally, even if the vegetable meals shown in Table 3 have quite high protein ADCs, they are usually poor protein sources compared to animal meals and therefore higher amounts of feedstuff are required to obtain the same amount of digestible protein, which can prove to be unsustainable.

Some researchers have already successfully replaced the totality of dietary fishmeal by plant meals with no reduction of growth performance in some fish species like rainbow trout, using canola protein concentrate or soy protein concentrate (Kaushik et al., 1995; Slawski et al., 2013), in blunt snout bream (*Megalobrama amblycephala*), using rice protein concentrate

as fishmeal replacement (Cai et al., 2018) and in Senegalese sole (*Solea senegalensis*), provided crystalline amino acids are supplemented to ensure requirement levels (Silva et al., 2009).

In European seabass, almost total fishmeal replacement has been successfully achieved by using plant protein sources. This is usually done by combining different plant proteins in order to balance the amino acid profile of the diet to be closer to seabass' requirements, instead of using a single plant protein source. For instance, Kaushik et al. (2004) have reduced to 2% the inclusion of dietary protein from fishmeal by using a combination of corn gluten meal, wheat gluten, soybean meal and rapeseed meal. However, such diet required L-Lysine and phosphorus to fulfill seabass' requirements. Moreover, this study also showed that the cost of producing the feed with the lowest fishmeal level would be similar to that of the fishmeal-based diet, probably due to the elevated price of the required supplements. In this study, the diet with lowest dietary fishmeal level (50 g/kg) was able to induce a growth performance similar to the fishmeal-based diet, although protein efficiency ratio was decreased and there was an increase in whole-body fat composition (Kaushik et al., 2004). This increased body fat indicates that dietary protein sources can affect lipid metabolism and fat deposition in European seabass, which was also highlighted by Dias et al. (2005) in another study. In that study, total fishmeal was replaced by three different single plant protein sources – two soy protein concentrates from different origins and corn gluten meal. L-methionine was supplemented in all plant protein diets and L-arginine and L-tryptophan were also supplemented in the corn gluten meal diet. However, even with free amino acid supplementation, growth performance was impaired with all plant protein diets, resulting in lower final body weights and protein efficiency ratios. This reduced growth performance might be explained by the available phosphorus in those diets, which was below the seabass requirements. Moreover, the influence of vegetable diets on lipid metabolism varied among studies. Kaushik et al. (2004) reported an increased body fat composition with vegetable diets, whilst Dias et al. (2005) showed that lipid gain was highest for the fishmeal-based diet. This study also shows that the different protein sources had a very different impact on the liver lipid fraction composition, plasma cholesterol and triacylglycerol levels as well as lipogenic enzyme activities, indicating a clear effect of the dietary protein source on the lipid metabolism (Dias et al., 2005).

Wheat gluten has been tested as single ingredient for partial fishmeal replacement in seabass diets (Tibaldi et al., 2005). In this study, two diets were formulated to replace 50 and 70% of the fishmeal. L-lysine and L-methionine were supplemented to both diets, and L-arginine was supplemented to the diet with the highest replacement level, while phosphorus was not supplemented in any diet. Growth performance remained unaffected with 50% FM

replacement, but final body weight decreased significantly with the 70% diet, probably as a result of reduced available dietary phosphorus, since the dietary amino acids had been balanced.

The replacement of FM by differently processed soybean meals has also been assessed on the growth performance, nutrient utilization and intestinal brush border enzyme activity of European seabass (Tibaldi et al., 2006). This study tested three different soybean processing methods used either as single substitutes or a combination of two of them to replace FM. In these experimental diets, DL-methionine was supplemented, but no phosphorus was added. 25% FM was successfully replaced by a standard dehulled-toasted and solvent-extracted soybean meal, while 50% FM replacement without growth performance impairment was possible with an enzyme-treated soybean meal with reduced oligosaccharide and trypsin inhibitor contents. The mixture of those two was however unable to replace 60% FM without impairing seabass growth performance. Brush border enzyme activity, however, had little differences among dietary treatments, indicating that the reduced digestibility and retention obtained with the different diets might have another genesis (Tibaldi et al., 2006).

Vegetable ingredients as lipid sources for fish

The total replacement of fish oil has also already been achieved without growth impairment in some species like Atlantic salmon, using rapeseed oil as FO replacement (Bell et al., 2001), and in rainbow trout, using either linseed oil as a single FO replacement or a 65:35 mixture of canola and linseed oil (Drew et al., 2007; Turchini & Francis, 2009).

In European seabass, similarly to what is seen in FM replacement studies, the research towards FO replacement has been conducted mainly using vegetable sources. Some studies use mixtures of different vegetable oils to mimic the fatty acid profile of FO (high PUFA and particularly n-3 PUFA levels), while other authors have studied vegetable oils as single FO substitutes in seabass diets. Overall, the dietary lipid source seems to affect the tissue composition more than the growth performance. Izquierdo et al. (2003) have successfully replaced 60% dietary fish oil with a mixture (1:3:6) of soybean, rapeseed and linseed oils without impairing growth performance but affecting the muscle fatty acid profile. The muscle fatty acid profile reflected that of the diet, resulting in reduced levels of important fatty acids like EPA and DHA, however, sensorial quality attributes of the final fillets were not affected (Izquierdo et al., 2003). Two blends of rapeseed, palm and linseed oils, at either 1:1.47:3.41 or 2:1:2, were used to replace 60% FO but resulted in decreased body weight after a 64-week feeding period in fish fed the mix with more linseed oil (Mourente & Bell, 2006; Mourente et al., 2005a, 2007). However, a finishing fish-oil based diet fed for 12 additional weeks was able to reverse that effect (Mourente & Bell, 2006). Using these vegetable blends did not compromise

non-specific immune function, intestine histology or the desaturation/elongation and h-oxidation activities of 14C-LNA and 14C-EPA in isolated hepatocytes and pyloric caeca enterocytes (Mourente et al., 2005a, 2007). More recently, a different blend (2:3:5) of those same oils (rapeseed, palm and linseed oils) has been successfully used to fully replace dietary FO without affecting growth performance, nutrient retention and nutrient digestibility in seabass (Castro et al., 2015a, 2015b). However, the lipid source alteration resulted in the up-regulation of hepatic genes related with cholesterol metabolism.

Soybean oil has successfully used to replace 50% dietary FO without negative effects on seabass growth, liver histology and tissue lipid composition, with lipids being preferentially deposited in the viscera regardless of the dietary treatment (Figueiredo-Silva et al., 2005). Rapeseed oil, soybean oil, linseed oil and olive oil have also been tested as single lipid sources to replace 60% FO in seabass diets without impairing growth performance (Izquierdo et al., 2003; Mourente & Dick, 2002; Mourente et al., 2005). Even though the fillet fatty acid profile was affected by the dietary lipid source, reference levels were achieved for most fatty acids after an additional 12-week feeding period with a finishing fish oil-based diet (Mourente et al., 2005), and no differences were noted among sensorial quality attributes of the final fillets of dietary treatments (Izquierdo et al., 2003). More recently, rapeseed oil has been successfully used to completely replace FO by Yılmaz and Eroldoğan (2015). In that study, total FO replacement has been achieved without impairing growth performance by using rapeseed oil or cottonseed oil as single replacement in seabass diets (Yılmaz & Eroldoğan, 2015) reporting higher body weight with rapeseed oil than with cottonseed oil.

Algae and single cell proteins

Alternative protein and lipid sources being increasingly tested and used as fishmeal and fish oil replacements include macroalgae, single cell products (from bacteria, yeasts, microalgae and protists) and plankton (FAO, 2018b; Rana et al., 2009). These ingredients can be rich sources of protein, fatty acids including omega-3 (e.g. in plankton), attractants and pigments to use in aquafeeds. Nevertheless, the production cost of these ingredients is still an issue as the resulting market price would be too high for aquafeed producers (FAO, 2018b), and, on the other hand, the exploitation of wild stocks would have negative impacts on the marine organisms' food chain (Rana et al., 2009).

Single cell protein sources like brewer's yeast have also been studied as fishmeal replacement in seabass diets, having successfully replaced 50% of dietary fishmeal protein without compromising growth performance, as long as dietary L-methionine was supplemented (Oliva-Teles & Goncalves, 2001). In fact, the inclusion of brewer's yeast up to 30% fishmeal replacement resulted in improved feed efficiency and protein utilization, indicating that at low levels this protein source can be a beneficial addition to seabass diets. Moreover, Santacroce et al. (2012) observed an antiperoxidative effect and no decreased growth in seabass juveniles fed diets with yeast replacing 20% of fishmeal protein. Yeast has also been used in seabass diets as supplements (at 0.08 and 0.8% of the diet), resulting in improved growth performance and intestinal health status (Rawling et al., 2019).

Macroalgae have been tested as FM replacements in several fish species, with different results according to the algae and the fish species. In seabass, *Gracilaria bursa-pastoris* and *Ulva rigida* replaced 17% FM without impairing growth performance, nutrient utilization or body composition, while that was only possible up to 8% FM replacement with *Gracilaria cornea* (Valente et al., 2006).

Microalgae can be very interesting feed ingredients for fish, improving fish nutritional value and omega-3 fillet content, growth performance and disease resistance, whilst decreasing nitrogen release into the environment (Becker, 2004). Furthermore, microalgae can be grown in a myriad of habitats, have simple nutritional requirements and very fast biomass production, they can accumulate useful metabolites, and their availability does not depend on fishmeal (Hemaiswarya et al., 2011). Several microalgae species have been tested in different fish species, as either protein sources, replacing dietary FM (Table 4), as lipid sources, replacing dietary FO (Table 5) or as feed additives, namely as sources of supplemental carotenoids (Table 6).

Table 4. Microalgae used as dietary fishmeal replacement in several fish species.

Microalgae species	Fish species	% FM replacement	Effects of microalgae biomass
Nannochloropsis sp. and *Isochrysis* sp. [1]	juvenile Atlantic cod (*Gadus morhua*)	15%	Feed intake and growth improved in the fish. No differences in survival, feed conversion ratios, and omega-3 and omega-6 fatty acids in the muscle among the treatment groups.
Arthrospira sp. [1]	Golden barb (*Puntius gelius*)	20%	Significantly increased growth rates of fish.
Nanofrustulum sp. [1]	Atlantic salmon, common carp (*Cyprinus carpio*)	5 or 10%	Growth performance and feed utilization did not exhibit any differences compared with fish meal-based feed, indicating algal meal as an effective replacement of fish meal.
Arthrospira maxima [1]	Red tilapia fingerling (*Oreochromis* sp.)	Up to 30%	No negative impact on growth performance.
Tetraselmis suecica [1]	European seabass	Up to 20%	No negative impact of the growth performance and major quality traits of fish.
Arthrospira sp. [1]	Tilapia (larvae/juveniles) (*Oreochromis* sp.)	Up to 43%	No negative impact on growth or feed intake and had a better FCR than a corn-gluten meal control.
Arthrospira sp. [1]	Parrot fish (*Oplegnathus fasciatus*)	5%	Significantly higher weight gain, protein efficiency ratios, feed intake, and lower feed conversion ratios than the fishmeal control.
Navicula sp., *Nannochloropsis salina* [1]	Juvenile red drum (*Sciaenops ocellatus*)	up to 10%	No any adverse effect found on the growth performance.
Spirulina sp. [1]	Rainbow trout	7.5%	Highest weight gain observed.
Scenedesmus almeriensis [1]	Gilthead sea bream (*Sparus aurata*)	38%	The inclusion of algae meal did not affect the feed intake.
Isochrysis sp. [2]	European seabass	20%	No adverse effect, feed intake or growth performance; greenish skin pigmentation, no differentiation among experimental diets on sensory analysis.
P. tricornutum [1]	Atlantic salmon	6%	No negative effect on growth, feed conversion of protein, lipid, energy, ash, growth performances, etc.
Nannochloropsis oceania [3]	Atlantic salmon	14%	No negative effects on growth performance and nutrient utilization.
Nannochloropsis sp. [4]	European seabass	31%	No negative effect on growth performance, whole-body composition and intestinal histological morphology.

1 - Reviewed by Shah et al. (2018); 2 - Tibaldi et al. (2015); 3 - Sørensen et al. (2017); 4 - Valente et al. (2019).

Table 5. Microalgae as fish oil replacement in fish feeds. Adapted from Shah et al. (2018)

Microalgae species	Fish species	% FO replacement	Effects of microalgae biomass
Nannochloropsis sp. and *Schizochytrium* sp.	Olive flounder (*Paralichthys olivaceus*)	100%	No negative effects on growth, feed efficiency or nutritive quality.
Schizochytrium sp.	Atlantic salmon	Up to 5%	No signs of toxicity, stress, inflammation, or any other negative effects of supplementation in diets; fillet quality good.
Isochrysis sp.	European seabass	Up to 36%	No adverse effect, feed intake or growth performance relative to controls.
Pavlova viridis, Nannochloropsis sp.	European seabass	50–100%	No negative effects on the growth performance and nutrient utilization of fish.
Schizochytrium sp.	Nile tilapia (*O. niloticus*)	100%	Improved weight gain, feed conversion ratio, and protein efficiency ratio and no significant change in survival rate.

Microalgae can have multiple beneficial effects and could potentially reduce or even replace fishmeal and fish oil, but there are still some challenges and disadvantages in using these ingredients, namely the high production costs (Becker, 2007; Sarker et al., 2016), low digestibility (Skrede et al., 2011), and the risk of contamination that could compromise an entire biomass production (Hannon et al., 2010). Hence, microalgae biomass has been mainly targeted as an additive, focusing on its benefits as a supplement rather than as a macronutrient source (Shah et al., 2018).

Table 6. Microalgae in fish feeds as a dietary supplement. Adapted from Shah et al. (2018)

Microalgae species	Fish species	Dietary inclusion level	Effects of microalgae
Arthrospira platensis	Red tilapia	30% inclusion in feed as carotenoid supplement	Improved fish color
Nannochloropsis gaditana, *Tetraselmis chuii*, *Phaedodactylum tricornutum*	Gilthead seabream	0.5 and 1% inclusion in feed	Enhanced defense activity
A. Platensis	Common carp	7.5% inclusion in feed as carotenoid supplement	Improved pigmentation
A. Platensis	Nile tilapia	0.5 to 2% inclusion in feed	Improved fish health conditions by tissue protection and antioxidant effects
A. platensis	Rainbow trout	10% inclusion in diet	Can be used as an immunostimulant

Agro-food by-products

The agro-food industry generates large amounts of vegetable and animal by-products that are often discarded. The inappropriate disposal of these by-products is responsible for environmental pollution and for the loss of useful biological resources (NRC, 2011). Recently, in light of the Circular Economy concept (European Commission, 2014), the European Commission has established initiatives to valorize the nutrients of these by-products that are considered "food no longer intended for human consumption" – meaning that for commercial reasons it is no longer intended for human consumption, but that was manufactured for human consumption according to EU food legislations and can hence be considered safe to use in animal nutrition. This valorization of agro-food by-products should produce valuable and sustainable novel sources of protein and lipids for fish feed, avoiding their use to produce compost, biogas or being disposed by landfill or incineration. However, due to the bovine spongiform encephalopathy (BSE) crisis in the 1990's, animal by-products have been considered potential sources of risk for public and animal health and, in the EU, land animal protein meals have even been banned from being used in animal feeds in 2001 (European Commission, 2001), with the exception of pet food and feeds for fur-producing animals. Nevertheless, some land animal meals have been re-allowed in some animal feeds, such as fish feed, since 1 July 2013, as long as they are considered low risk material and properly rendered before being used as feedstuffs (European Commission, 2013).

In the European Union, it has been estimated that about 222 million tons of food waste and by-products are produced every year, with the majority of these by-products coming from the vegetable and fruit industry, followed by milk and meat production (AWARENET, 2004). In the animal by-product category, for instance, 25 to 42% of each slaughtered animal is not intended for human consumption, and almost 2.5 million tons of fallen stock are collected every year (EPFRA, 2016). While a significant amount of these by-products is already valorized and re-introduced into the economy (i.e. as animal feed ingredients), large amounts are still discarded by landfill or by other means (Waldron, 2009).

Using a variety of cooking and drying processes, animal by-products can be rendered into valuable protein and lipid sources for animal feeds (Meeker & Hamilton, 2006) that due to their amino acid profile are usually more suitable than vegetable meals to replace FM (NRC, 2011). But, animal fats, like vegetables, have low or no levels of important omega-3 fatty acids like EPA and DHA, which complicates total fish oil replacement (Trushenski & Lochmann, 2009). Since the use of non-ruminant land animal meals in European aquafeeds has been re-approved (European Commission, 2013), these ingredients have once again gained some interest as aquafeed feedstuffs for European species. In 2011, it was estimated that the use

of these ingredients as both protein and lipid sources within compound aquafeeds ranged between 0.15 and 0.30 million tons (< 1% total global aquafeed production; Tacon et al. (2011)). There is still considerable room for an increase in the use of these feedstuffs in aquafeeds, but the consumers' acceptance may play a key role on that increase. On the other hand, the amounts of fishmeal and fish oil obtained from fisheries by-products are increasing, and it was estimated that about 25-35% of the fishmeal and fish oil production already comes from by-products (EUMOFA, 2018; FAO, 2016). Also, since strategies are being implemented to reduce and stop discarding fisheries by-catch products, this percentage should increase (EUMOFA, 2018; Viðarsson et al., 2015). Protein and lipid sources from rendered fisheries by-products are considered the best replacements for the traditional fishmeal and fish oil, due to their nutritional characteristics. However, proper treatment must be applied in order to assure absence of pathogens and contaminants (Hardy et al., 2005).

The food and feed ingredients rising price is a tendency most likely to remain, due not only to the increasing population size and respective food demand, but also to the trend to use feedstuffs to produce biofuel, the increased production and transport costs, and changes in global trades (FAO, 2018b; Rana et al., 2009). On the other hand, the use of the carbohydrate-rich fraction of vegetable sources to produce biofuels can create opportunities to use the protein fractions as feed ingredients (Hauptman et al., 2014). While most of the research has been conducted towards decreasing the use of fishmeal and fish oil in aquafeeds, the continued and increasing use of vegetable meals, oils and carbohydrate sources can also represent an obstacle to the sustainable future of aquaculture, since non-carnivorous species represent a significant portion of aquaculture production (Tacon et al., 2011). Thus, the sustainability of aquaculture and aquafeeds should rely on searching for other nutrient sources that cannot compete with other economic sectors, and also on maximizing the use of available locally produced feedstuffs, whose production may sustainably grow along the aquafeed sector.

The search for new ingredients has therefore a double interest – the environmental sustainability of the sector and the international competitiveness of local enterprises in the global market. The inclusion of locally produced ingredients could significantly reduce the carbon footprint and consequently the environmental impact of aqua feeds and may even have important repercussions for both the companies that produce and the ones that use such feedstuffs. However, there is little information available on the use of locally produced agro-food by-products as feedstuffs in practical diet formulations for European seabass. This gap in literature is partly due to the prohibition of using land animal-origin ingredients in 2001 in aquafeeds (European Commission, 2001), warranting new studies.

Description and legislation

Animal by-products are defined by The Commission of the European Communities Regulation (EC) No. 1096/2009 as the whole body of an animal, parts of it, or products derived from animals that are not meant for human consumption (European Union, 2009). Based on their level of risk of pathogens and toxic substances transmission, these by-products are divided into one of three categories:

Category 1 is very high risk material and should be buried or incinerated at specific approved treatment plants. Briefly, this category includes:

- entire bodies and body parts of all animals suspected of or actually infected with a transmissible spongiform encephalopathy (TSE);
- any animal that was not farmed or wild (pets, zoo animals, …);
- animals used in experimentation;
- wild animals whenever suspected of being infected with transmissible diseases to other animals or humans;
- animal tissues of specified risk;
- catering waste obtained from international transport;
- animal tissue collected during waste water treatment from Category 1 processing plants;
- mixtures of Category 1 material with materials from either of the two other categories.

Category 2 is high risk material, but that can be used as raw material for composting, biogas generation and energy production, as long as it is properly pretreated at high temperature and pressure conditions. This category mainly includes:

- manure and digestive tract content;
- animal tissue collected during waste water treatment from Category 2 processing plants;
- whole animals or parts of non-ruminant animals that died other than being slaughtered for human consumption, including those killed for disease control purposes and those that are slaughtered to prevent disease propagation;
- products of animal origin imported from a third country or dispatched to another Member state that fail to comply with the respective Community veterinarian legislation;
- Mixtures of Category 2 material with Category 3 material.

Category 3 is low risk material. This category comprises parts of healthy animals that would be fit for human consumption but are, however, not intended for human consumption

for commercial reasons, being permitted to be used for certain beneficial purposes such as the incorporation in animal feeds or even for the extraction of interesting compounds. This category mainly includes:

- Feathers, skin, hides, blood, placenta, heads, feet, horns, hoofs and raw milk obtained from healthy animals at slaughterhouses;
- Shells from shellfish or eggs;
- Aquatic and terrestrial invertebrates (other than species pathogenic to humans or animals);
- Carcasses and parts of slaughtered animals that are fit for human consumption but are not intended for human consumption for commercial reasons.

> *For a more comprehensive list of all by-products included in each category, please see the Regulation (EC) No 1069/2009 of the European parliament and of the council of 21 October 2009, regarding the rules regarding animal by-products to control and prevent TSE (European Union, 2009).*

The only animal by-products allowed in animal nutrition are, therefore, those classified as Category 3 material. Nonetheless, proper treatment can be necessary before being considered eligible as feedstuffs, assuring that safe ingredients are produced and creating opportunities to add more value to those by-products. The methods approved to process these animal by-products are detailed in the Regulation (EC) No 1774/2002 of the European Parliament and of the Council of the European Union (European Union, 2002).

Even after the BSE crisis and before many animal by-products were re-authorized in animal feeds in 2013, the use of rendered animal fats, including beef fat (or tallow), in animal feeds was not forbidden in the EU, as the pathogen (a prion) is related to the protein portion of the animal. According to the Scientific Steering Committee (SSC, 2001), there is no evidence that tallow derived from ruminant animals would constitute a TSE (transmissible spongiform encephalopathy) risk, since TSE agents are associated with protein impurities that may be present in the end-product. Therefore, tallow could be safely used in feed production (except for calf feed) and pet food, if it is purified to levels below 0.15% insoluble impurities.

Utilization in animal nutrition

Due to the change in legislation, the use of land animal by-products with industrial interest and capable of being used in animal feeds as a replacement for protein or lipid sources gained recent interest. Several by-products have been characterized regarding their nutritional properties and found to be potentially adequate raw materials in animal feeds (Gouveia, 1991). More recently, a number of ingredients resulting from poultry processing plants comprising poultry by-product meal (heads, legs, bones and viscera) and feather meal, were characterized for their chemical composition and identified as potential sources of protein (including antioxidant peptides), proteases and polyunsaturated fatty acids (Lasekan et al., 2013); fish protein hydrolysate (from sardinelle, *Sardinella aurita*, by-products) seems a good source of protein and of natural antioxidants (Bougatef et al., 2010); whereas shrimp (Sila et al., 2014) and snow crab (Beaulieu et al., 2009) by-product hydrolysates where identified as both good sources of peptides – with antioxidant activity – and of essential fatty acids. Many natural extracts from marine by-products were also described as exceptional nutritional components to be applied in the health and food sectors (Ferraro et al., 2010).

Agro-food by-products as protein sources for fish

Large amounts of agro-food by-products are produced in Portugal and in Europe but have been poorly evaluated in fish. Wheat germ, a by-product of the flour milling industry, has been described as an excellent source of proteins, lipids and bioactive compounds, therefore highly valued as a food supplement (Brandolini & Hidalgo, 2012); okara meal, a by-product obtained by processing soya beans to produce tofu or soya milk, was also identified as a good feed supplement for its high protein and fiber content (Li et al., 2012). Spent brewer's yeast (from the genus *Saccharomyces*) is the second major by-product from brewing industry and has also been pointed out as an important protein source with potential to replace fishmeal (discussed in "Algae and single cell proteins"). Some of the fractions obtained from agro-food by-products are applicable to human nutrition and pharmaceutic sectors, since they are known to have bioactive properties, such as hydroxyapatite obtained from fish bones and scales, that can be used in dental prosthetics and tissue regeneration, and hydrolyzed collagen obtained from the same by-product, which is known to have anti-hypertensive properties (Ferraro et al., 2010). However, the processes used to obtain these interesting fractions for humans also generate larger peptides with lower value for humans that could still have nutritional value for animal nutrition. Also, hydrolyzed keratin obtained from poultry feathers was reported to have antioxidant properties (Fakhfakh et al., 2011) and the peptide fractions obtained from spent brewer's yeast and from wheat germ can have anti-hypertensive and prebiotic functions (Brandolini & Hidalgo, 2012).

The potential of new ingredients as fishmeal substitutes in aquafeeds largely depends on each species' ability to digest them: selecting ingredients with high digestibility improves the performance of fish and reduces waste production. The processing method applied to by-products has a great effect on the nutrients' bioavailability of the final product and, therefore, its nutritional value. For instance, poultry by-products are usually treated by protein hydrolysis, but this can be done by heat and pressure, by applying enzymes (to achieve a higher degree of hydrolysis) or in bacterial cultivation, and each process generates a different mixture of free amino acids and peptides of varying length and bioactive properties (Fontoura et al., 2014). Even when using the same type of hydrolysis, the duration, temperature and pH of the process can also affect the quality and composition of the final product (Lasekan et al., 2013). Poultry by-product meal has shown high protein digestibility levels in fish species, varying from 78 to 86% in marine species such as European seabass, Atlantic cod, gilthead seabream and turbot (*Psetta maxima*; Davies et al. (2009); Tibbetts et al. (2006)), and ranging from 83 to 96% in Coho salmon (*Oncorhynchus kisutch*) and rainbow trout (Bureau et al., 1999; Cheng & Hardy, 2002; Cheng et al., 2004; Sugiura et al., 1998). Hydrolyzed feather meal protein digestibility largely varies among fish species, ranging from 22 - 79% in gilthead seabream, turbot, Atlantic cod, European seabass and rockfish (*Sebastes schlegeli*; Davies et al. (2009); Lee (2002); Tibbetts et al. (2006)), and from 77 to 86% in Coho salmon and rainbow trout (Bureau et al., 1999; Cheng et al., 2004; Sugiura et al., 1998). Wheat by-products, such as wheat middlings and wheat gluten, have shown a 92-100% protein digestibility in hybrid striped bass (*Morone saxatilis*), red drum and Atlantic cod (McGoogan & Reigh, 1996; Sullivan & Reigh, 1995; Tibbetts et al., 2006) and from 86 to 91% in Coho salmon and rainbow trout (Sugiura et al., 1998). The protein digestibility of brewer's yeast has also been evaluated in some fish species, reporting 73% in juvenile rockfish (Lee, 2002), 57% in rainbow trout (Cheng et al., 2004) and 92% in bluntnose black bream (*Megalobrama amblycephala*; Zhou et al. (2008)).

Land animal by-products as lipid sources for fish

In fish culture, dietary lipids play an important role not only as sources of essential fatty acids but also to provide energy and a protein-sparing effect in many fish species. PUFA are present in high quantity in fish oils, being susceptible to oxidation, while land animal fats, such as pork lard, beef tallow and poultry fat are richer in SFA and MUFA that are more resistant to auto-oxidation, which, in turn, makes them more stable ingredients (Watanabe, 1989). Besides this, poultry fat also has a higher content of *n*-6 PUFA and even though it has a lower amount of *n*-3 PUFA it has more alpha-linolenic acid (18:3*n*-3) than fish oil (Emery et al., 2014). On the other hand, the energy and fatty acids availability of these ingredients highly depends on their digestibility, which has been shown to decrease in ingredients that are rich in SFA (Hua & Bureau, 2009). Some studies have already been conducted regarding the use of land animal

by-products as replacements for fish oil. Poultry oil has been reported to have a reduced digestibility of monounsaturated (MUFA) and polyunsaturated fatty acids when compared to fish oils in Atlantic halibut (*Hippoglossus hippoglosssus*), even though the total lipid ADC of the diet containing poultry oil was similar to the diet with fish oil (Martins et al., 2009). This ingredient can fully replace fish oil in diets for barramundi (*Lates calcarifer*) without impairing fish growth. But, such diets resulted in reduced level of *n*-3 PUFA and increased levels of *n*-6 PUFA and total MUFA of the fish fillet, leading to a reduced peroxidation index and increased fillet shelf life (Ahmad et al., 2013). Caballero et al. (2002) reported reduced lipid digestibility of swine lard in rainbow trout compared to fish oil. Likewise, beef tallow was used as a replacement of poultry oil in diets for Atlantic salmon also inducing decreased lipid digestibility (Emery et al., 2014).

Although there isn't much information regarding the digestibility of land animal fats, their effects as fish oil replacements on growth performance and muscle fatty acid composition were reported in several studies. On Japanese seabass (*Lateolabrax japonicus*), poultry fat, pork lard and beef tallow replaced 50% of dietary fish oil with no effects on growth performance or on whole body composition, but lipid content in the liver increased in the fish fed either tallow or poultry fat (Xue et al., 2006). Contrarily, a study conducted in largemouth bass (*Micropterus salmoides*) with total replacement of fish oil by poultry fat or beef tallow, reported a lower level of liver lipids in the fish fed diets with tallow. This diet also led to a reduced growth and increased feed conversion ratio, while the diet with poultry fat used in the same study led to an increased specific growth rate compared to the fish fed the diet with fish oil (Yun et al., 2013). On the other hand, while beef tallow increases the amounts of SFA in the muscle of rainbow trout (Bayraktar & Bayir, 2012), the use of poultry fat seems to decrease SFA and increase the amount of muscle *n*-6 PUFA in barramundi, yellowtail kingfish, Atlantic salmon and largemouth bass (Ahmad et al., 2013; Bowyer et al., 2012; Friesen et al., 2015; Yun et al., 2013), compromising its nutritional value and hence the cardiovascular health benefits of eating fish. However, according to Friesen et al. (2015) such alterations in the fatty acid profile of the muscle when using poultry fat could be restored, after a finishing period with a fish oil-based diet, to values similar to those observed in fish fed the fish oil diet. In light of these findings, and even though, as previously mentioned, this differential fatty acid profile may lead to a more stable final product, a fatty acid supplementation or a finishing period with a fish oil diet would probably be recommended in diets where these replacements are made, to assure that the final fillet still provides important fatty acids to the consumer.

Land animal fats have been tested in some important fish species, but to our best knowledge, there are no studies conducted in European seabass using such lipid sources as fish oil replacement.

Life Cycle Assessment

LCA methodology: what is it and what is it used for?

The concept of life cycle assessment (LCA) started being developed in the late 1960s in the USA, and the formal analytical scheme that defines it was first conceived in 1969 by Harry E. Teasley, Jr. (Hunt & Franklin, 1996). At that time, The Coca-Cola Company was looking at several issues related with the manufacture of their own packaging, regarding the use of either refillable glass bottles or disposable plastic bottles or aluminum cans. To help with this decision, they wanted to analyze several aspects of the alternatives considered, including the environmental consequences of using different materials, manufacturing and use of packaging. While managing this study, Teasley considered including the energy, which at the time was not being thought of as an ecological issue (Hunt & Franklin, 1996). Due to confidentiality issues, the results of this study were never published, but they were used by the company to make informed decisions on their packaging. Not long after that, the academic world in the UK caught up with this methodology, and in the early 1970s the oil crisis led to a boost in the methodological development of LCA.

Despite of the evolution of the methodology, until the late 1980s there was not much public interest in LCA studies, as they were mainly driven by private companies to evaluate the use of different materials and most of the results obtained were kept as a company secret. However, in 1988 there was a sudden rise of environmental consciousness in the US which brought LCA to the attention of several public and private organizations, ultimately leading to the creation of the first workshop on the topic, organized by the Society of Environmental Toxicology and Chemistry (SETAC) in 1990. It was only after this workshop that the term LCA started being used (until then it was designated REPA – Resource and Environmental Profile Analysis) and the first LCA presentation and guidelines were published (Heijungs et al., 1992; Hunt et al., 1992; SETAC, 1993). Afterwards, several methodological standards have also been published by the International Organization for Standardization, and nowadays LCA is considered a very important decision-support tool not only for industries seeking cleaner production but also for policy makers as a foundation for sustainable development.

But what is, in fact, LCA?

LCA is defined, in ISO 14040, as a “compilation and evaluation of the inputs, outputs and potential environmental impacts of a product system throughout its life cycle” (ISO, 2006). Therefore, it is a tool to analyze the environmental burdens of a certain product, for which the “product system” incorporates all the processes involved in the life cycle of a product, including raw material extraction, manufacturing, use and final disposal, which is also known as a “cradle

to grave" analysis. This provides a holistic approach that incorporates all the environmental impacts, regardless of where in the chain they occur, into a single framework. This is important because since the consumption of the final product is the driving force of economy, it offers great opportunities for environmental management along a chain of processes related to any product (Guinée et al., 2002). On the other hand, another very important reason for the use of this "cradle to grave" approach is that it avoids "problem shifting", since the whole life cycle is being analyzed, instead of just a portion of it. When trying to reduce environmental impacts, it is very important to look at the outcomes in the whole production system instead of focusing in just one point of the life cycle – reducing the impacts in a single production phase could potentially lead to increased impacts in another phase. For instance, producing a car based in aluminum instead of steel can decrease the consumption of fuel in the use phase of the vehicle, however, aluminum production requires more energy consumption than steel production. Therefore, all these facts have to be considered to evaluate if using aluminum is in fact more environmentally sustainable than using steel to produce cars (Guinée et al., 2002).

LCA can be applied to identify the origin of environmental impacts associated with a certain product, compare the impacts of a product when considering different production variables, designing eco-friendly products and choosing among alternative comparable products. Thus, LCA can be used to help businesses and policy makers take informed decisions when trying to consider environmental benefits.

The life cycle assessment methodology has been standardized and is detailed in ISO 14040:2006 (ISO, 2006). According to the ISO standard, a complete LCA must be composed of four stages, which can interact with each other at any time of the LCA process: goal and scope definition, inventory analysis, impact assessment and interpretation (Figure 10).

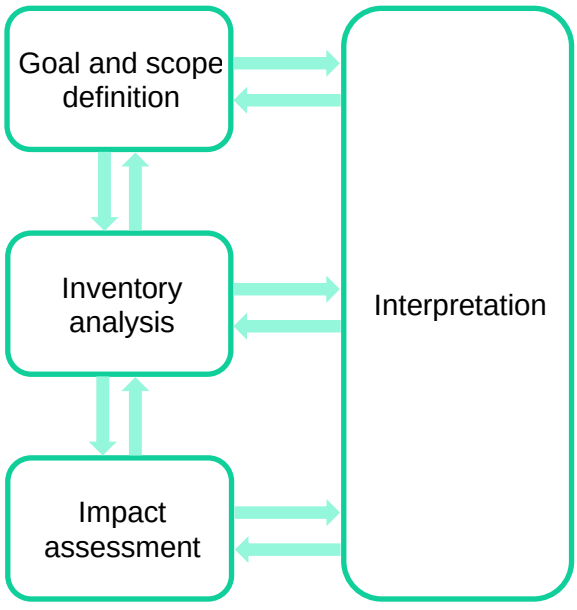

Figure 10. Life cycle assessment framework, adapted from ISO 14040:2006 (ISO, 2006).

The first LCA stage, goal and scope definition, is where the purpose of the study and its applications are defined, as well as the system boundaries, function and functional unit. The "system boundaries" of an LCA study define which processes of the product's life cycle are to be included in the study, the "function" defines what is provided by the product system, and the "functional unit" is a quantified description of the product system's

performance (ISO, 2006). All of these must be established in this initial stage, as the function and functional unit will define the reference to which the inputs and outputs are related and therefore are necessary to allow comparability of LCA results among different studies (ISO, 2006). The system boundaries, on the other hand, define which unit processes should be included within the LCA – the criteria used to establish the boundaries, as well as any assumptions made, should be identified and justified in the scope of the study as well (ISO, 2006). However, since LCA is an iterative technique, adjustments to the scope of the study may be required while the study is being conducted and information is being collected.

The second stage of LCA, the life cycle inventory (LCI), is probably the hardest and more resource and time-consuming phase of this analysis. This stage involves the collection of data and calculations used for the quantification of relevant inputs and outputs of a production system. Inputs and outputs mainly include the use of natural resources (including energy) and emissions to air, water and land associated with the system. Even though databases of several material LCI are already available for consultation from previous studies, when conducting an original study, it is important to obtain specific production data from private companies or from specific users, so that the life cycle is modeled as close as possible to the reality in study. During this phase, some important calculations must be established and taken into consideration, namely the use of allocation methods. These are required whenever the production system considered is multi-functional, i.e. produces more than just one final product (including by-products or co-products). To conduct an LCA on a multi-functional system, the materials and energy intake, as well as the resulting environmental emissions must be partitioned among the different resulting products according to clearly defined allocation methods. This way, we can calculate the impacts generated in a production system that are associated with just one of its products. Even though international methodological standards exist, allocation decisions are still a target of debate and inconsistency in LCA studies (Azapagic & Clift, 1999). According to ISO 14040 (2006), it is preferable to use system expansion or to sub-divide the system process to isolate the product of interest. System expansion involves the modeling of alternative production systems for each co-product of the studied system and giving credit to the impacts of those alternative systems compared to the total impacts of the multi-functional system. This way, it would be possible to isolate the portion of environmental impacts attributable to the product under study. However, the sub-division of many processes is not possible, and it is rarely viable to perform system expansion due to the inexistence of alternative product systems or because of time or data limitations. The next best option recommended by the ISO standard is to partition the environmental burdens of the multi-functional system according to specific relationships among the co-products, like mass, relative economic value, energy content, etc. (Ekvall & Finnveden, 2001). Ayer et al. (2006) have a

more detailed study on the allocation problems and solutions used for seafood production systems LCA studies. This stage is also iterative, since while data is collected and more is learnt about the system, more data requirements may be defined and even goal or scope revision may be necessary.

From the results obtained in the LCI phase, the life cycle impact assessment (LCIA) is conducted, “associating inventory data with specific environmental impacts and attempting to understand those impacts” (ISO, 2006). Usually, in this stage, specific LCA software programs are used to transform the LCI data into quantitative environmental impact contributions. The selection of the impact categories to be evaluated in this phase will depend on the goal and scope of the study. Since the methodological and scientific background for LCIA is still being developed, there is subjectivity in this phase and therefore transparency is essential to allow a clear description and report of assumptions (ISO, 2006).

The final stage of an LCA study is the life cycle interpretation – in this phase, all the findings from LCI and LCIA are combined along with the goal and scope of the study, in order to find conclusions and provide recommendations to decision-makers. This information can then be used to compare competing production technologies or products regarding their environmental performance, and, more specifically, to provide recommendations on how to improve that environmental performance. Once again, according to ISO 14040, this stage is also iterative, as it may promote the revision of the LCA scope and the nature and quality of the data collected for the inventory (ISO, 2006).

Although the initial LCA studies were aimed at packaging development and product design (Hunt & Franklin, 1996), they have evolved into other areas such as waste management – mainly comparing the environmental burdens of different waste management options (Barton et al., 1996; Cherubini et al., 2009; Lundie & Peters, 2005; Zang et al., 2015) and food production systems, including both process-specific and product-specific studies. Even though most food production systems have focused on agricultural systems, recently, LCA studies on fisheries and aquaculture production have increased (Mattsson & Ziegler, 2004). However, this methodology was not applied to the production of aquafeed until recent years (Papatryphon et al., 2004). Studying the LCA of aquafeeds is a major concern, particularly when the main goal is to reduce environmental impacts by replacing fishmeal and fish oil with more sustainable feedstuffs. However, the number of papers on this subject is still very low (Boissy et al., 2011; Davis et al., 2010; Pelletier & Tyedmers, 2007a; Samuel-Fitwi et al., 2013).

LCA of feed ingredients and food products

With increased concern about the sustainability of food production and consumption, several advances have been made towards the use of LCA to characterize activities related with food products, including the production processes, the food processing and the packaging, storage and distribution.

The production of wheat crops, which can also be used as feed ingredients, has been studied in a life cycle perspective, since crops can also be used as a raw material for bread production. LCA studies on bread go from crop production methods to different milling technologies, bread production processes and packaging (Braschkat et al., 2004; Van Holderbeke et al., 2004). These authors indicated a combined scenario of wheat production, industrial milling and a large bread factory as the most environmentally advantageous way to produce bread. Also, even though there are more differences between industrial and homemade bread production than between conventional and organic production, usually organic production requires more land area usage than the conventional one. The production phases with major impact in all categories analyzed were the transportation and cereal production, while the baking phase was mainly responsible for the impacts related with photo-oxidation and energy use.

Beer production is also a widespread industry which has already been studied to some extent, and it leads to the production of some by-products that can be used as ingredients in feeds. The highest impacts associated with beer production occur in the wort production, followed by filtration and packaging, and then by the fermentation stage and storage (Takamoto et al., 2004). In other studies, the production of packaging materials and respective manufacturing, as well as the harvesting and transport of the cereals used for beer production have also been reported as responsible for most of the emissions in these systems (Hospido et al., 2005; Koroneos et al., 2005). The different results among these studies are due to different choices of system boundaries, as the Takamoto study (2004) did not consider several processes taken into consideration in the other studies, such as transport of resources, beer containers and final product, waste treatment, and the recycling of glass bottles. This shows that clearly defining the system boundaries is very important, as different choices can lead to very different result interpretations.

In several European countries, the dairy industry has already been widely analyzed regarding its environmental impacts. Dairy production also leads to the production of by-products that can be used as feed ingredients, such as whey protein, and therefore the LCA of the dairy production has repercussions on the environmental impacts of using such by-products (Caugant et al., 1993; Kim et al., 2012; Sinha et al., 2007). For instance, studies show

that producing organic milk can reduce pesticide use and mineral surplus but increases the requirement for arable land when compared to conventional production (Cederberg & Mattsson, 2000; Williams et al., 2006). The studies by Williams and by Cederberg and Mattsson suggest measurements that should be implemented to both studied systems in order to reduce the impacts of milk production, such as reduced nutrient surplus and use of pesticides on the feed production, for the conventional system, and higher use of concentrated feed for organic production. Other studies have reported that the agricultural phase is the main environmental hotspot in the life cycles of milk and semi-hard cheeses (Berlin, 2002; Hospido et al., 2003), but the processes of packaging, waste management and cleaning can also have considerable impacts (Casey & Holden, 2004; Eide, 2002) and the main impact of these production systems is actually associated with the high water consumption, energy consumption and effluent discharges.

Several different agro-food ingredients that can be used in animal feeds, like rice, potato, tomato and sugar beet, have also already been subject of LCA studies (Roy et al., 2009). Most of these studies include the production and post-harvest phases of the production life cycles, and regardless of the product analyzed, results seem to be highly dependent on location, size and intensity of the cultivation, production method, and the crop variety cultivated. Results have also shown that the use of nitrogen fertilizers has a huge impact on the results, with land use being the major issue when low nitrogen was used, and eutrophication the major problem at high nitrogen rates. Using genetically modified varieties has also been shown to help decrease environmental impacts. For instance, in sugar beet, cultivating herbicide tolerant sugar beet would be less harmful for both the environment and human health than the traditional crop, as it can decrease emissions from herbicide manufacture, field operations and transport (Bennett et al., 2004). Additionally, as previously reported for other production systems, in agro-food production, shifting from conventional to organic production may reduce the use of fertilizers but increases land use.

Although as previously mentioned, agro-food by-products can be valuable feed ingredients, they usually need to be processed before being introduced in animal feeds and the processing methods require energy consumption and other resources, which, in term, have their own environmental impacts associated. Therefore, it is important to evaluate the life cycle of processed by-products being used as feed ingredients, including the processing phase of those by-products, in order to determine the environmental benefits of incorporating them in feeds. Hence, LCA studies have been implemented to assess the impacts of the valorization of several food production sectors by-products, like mussel (Iribarren et al., 2010), canned anchovy (Laso et al., 2016) and human food residues (Ogino et al., 2007, 2012). These studies indicate that the fuels used in the processing phase are the main responsible for the

environmental impacts generated (Iribarren et al., 2010; Laso et al., 2016), as well as the production of materials used for packaging final products (Laso et al., 2016). However, some of them compare the environmental impacts of valorizing these by-products versus their end of life by incineration or landfilling (or wastewater treatment in the case of rice-washing water) and conclude that their valorization is the more environmentally friendly option (Iribarren et al., 2010; Laso et al., 2016; Ogino et al., 2012).

Aquaculture and meat production systems have also been reported in LCA studies, and the environmental impacts have been reported as highly dependent on the feed type and production, length of feeding period, animal housing and manure management (Abdou et al., 2017; Aubin et al., 2009; Nunez et al., 2005; Ogino et al., 2004; Pelletier & Tyedmers, 2007; Samuel-Fitwi et al., 2012; Williams et al., 2006). Similarly to what was found in the aforementioned production systems, organic meat farming was also reported to reduce pesticide use but increases the land use, thus increasing the global warming potential impacts when compared to conventional production systems in the UK (Williams et al., 2006). While meat production LCAs rarely extend to the agricultural phase, studies that do cover that phase indicate it as the main source of impacts in the life cycle of meat products (Foster et al., 2006; Roy et al., 2008) and, for instance, the replacement of soya meal by pea and rapeseed meals in feeds have been suggested to decrease those impacts. Fish feed is the main contributor for most impacts studied in aquaculture production, not only due to the utilization of fishmeal and fish oil as feedstuffs but also due to poor feed conversion ratios that lead to nitrogen and phosphorus releases into the environment (Abdou et al., 2017). To avoid this, it is imperative to optimize diet production, by using more environmentally friendly ingredients and improving feed conversion ratios, and to promote better feeding strategies in farms, decreasing those emissions (Aubin et al., 2009).

As mentioned before, the selection of the functional unit when conducting an LCA can have a huge impact on the results. For instance, when considering protein as the functional unit, chicken production is reported to be more environmentally efficient than pork production, but the opposite happens when energy content is used as functional unit, while beef production is the least efficient system for both these scenarios (Roy et al., 2008). To highlight this, the LCAs of multiple livestock products has been reviewed by de Vries and de Boer (2010) and the results show the differences among location and according to different choices of functional unit (Table 7).

Table 7. LCA studies of several livestock products (adapted from de Vries and de Boer (2010)).

Production system	System type	Country	Functional Unit	GWP	Acidification		Eutrophication		Land	Energy
				kg CO_2 e	kg	unit	kg	unit	m^2	MJ
Pork	Conventional	NL	t protein	77,883	675	NH_3 e	2491	N e	55,000	397,252
	Good agricutural practice	F	kg live weight	2.3	0.044	SO_2 e	0.021	PO_4^{3-} e	5.4	16
	Red label	F	kg live weight	3.5	0.023	SO_2 e	0.017	PO_4^{3-} e	6.3	18
	Heavier finishing	UK	t dead weight	6080	391	SO_2 e	97	PO_4^{3-} e	6900	15,500
	Indoor breeding	UK	t dead weight	6420	507	SO_2 e	119	PO_4^{3-} e	7300	16,700
	Outdoor breeding	UK	t dead weight	6330	362	SO_2 e	95	PO_4^{3-} e	7500	16,700
	Conventional	UK	t dead weight	6360	395	SO_2 e	100	PO_4^{3-} e	7400	16,700
	Single intensive pig farm	S	kg bone-fat-free meat	4.8	2.6	mol H^+ e	2.0	O_2 e	15	22
	Conventional	NL	kg live weight	3.7	0.031	SO_2 e	0.018	PO_4^{3-} e	-	16
Chicken	Conventional	UK	t dead weight	4570	173	SO_2 e	49	PO_4^{3-} e	6400	12,00
	Free range	UK	t dead weight	5480	230	SO_2 e	63	PO_4^{3-} e	7300	14,500
	Conventional	F	t live weight	2079	35	SO_2 e	2.1	PO_4^{3-} e	5500	16,000
Beef	100% sucker	UK	t dead weight	25,300	708	SO_2 e	257	PO_4^{3-} e	38,500	40,700
	Lowland	UK	t dead weight	15,600	452	SO_2 e	153	PO_4^{3-} e	22,800	26,800
	Hill and upland	UK	t dead weight	16,400	510	SO_2 e	169	PO_4^{3-} e	24,100	29,700
	Non-organic	UK	t dead weight	15,800	469	SO_2 e	157	PO_4^{3-} e	23,000	27,800
	Specialist beef and dairy breeds	I	kg live weight	11	-	-	-	-	-	-
	Conventional, dairy calves	S	kg meat	17	4.2	mol H^+ e	3.4	O_2 e	33	40
Milk	Average farm	NZ	kg milk	0.93	0.0081	SO_2 e	0.0029	PO_4^{3-} e	1.2	1.5
	Single specialized farm	S	t ECM	990	18	SO_2 e	58	NO^{3-} e	1925	2800
	Production >7500 ECM/ha	S	kg ECM	0.87	0.010	SO_2 e	0.0038	PO_4^{3-} e	1.5	2.6
	Production <7500 ECM/ha	S	kg ECM	1.0	0.011	SO_2 e	0.0042	PO_4^{3-} e	1.9	2.7
	Intensive	G	t milk	1300	19	SO_2 e	7.5	PO_4^{3-} e	-	2700
	Extensive	G	t milk	1000	17	SO_2 e	4.5	PO_4^{3-} e	-	1300
	Average Irish dairy unit	I	kg ECM	1.3	-	-	-	-	-	-
	2 typical Galician dairy farms	ES	1 L packaged milk	1.1	0.0085	SO_2 e	0.0053	PO_4^{3-} e	-	6.2
	10 conventional commercial dairy farms	NL	kg FPCM	1.4	0.0095	SO_2 e	0.11	NO^{3-} e	1.3	5.0
	119 dairy farms	NL	kg FPCM	1.4	0.011	SO_2 e	0.12	NO^{3-} e	1.3	5.3
	Non-organic	UK	10,000 l milk	10,600	162	SO_2 e	63	PO_4^{3-} e	11,900	25,200
	More fodder as maize	UK	10,000 l milk	9800	164	SO_2 e	61	PO_4^{3-} e	11,800	23,600
	60% high yielders	UK	10,000 l milk	10,200	159	SO_2 e	60	PO_4^{3-} e	11,400	24,200
	20% autumn calving	UK	10,000 l milk	10,300	159	SO_2 e	65	PO_4^{3-} e	12,100	23,400
Eggs	Battery cage	NL	kg egg	3.9	0.032	SO_2 e	0.25	NO^{3-} e	4.5	13.0
	Deep litter	NL	kg egg	4.3	0.057	SO_2 e	0.31	NO^{3-} e	4.8	13.4
	Deep litter with outdoor run	NL	kg egg	4.6	0.065	SO_2 e	0.41	NO^{3-} e	5.7	13.9
	Aviary with outdoor run	NL	kg egg	4.2	0.042	SO_2 e	0.35	NO^{3-} e	5.1	13.7
	Non-organic	UK	20,000 eggs	5530	306	SO_2 e	77	PO_4^{3-} e	6600	14,100
	100% cage	UK	20,000 eggs	5250	300	SO_2 e	75	PO_4^{3-} e	6300	13,600
	100% free range	UK	20,000 eggs	6180	312	SO_2 e	80	PO_4^{3-} e	7800	15,400

Goals of this thesis

Fishmeal and fish oil, traditionally used as protein and fatty acid sources in aquafeeds, are considered environmentally unsustainable ingredients, not only for being produced from wild stocks of fish (depleting natural resources and competing with human nutrition), but also because they are usually imported from South America, having environmental impacts associated with their transportation. Replacing fishmeal and fish oil with by-products produced closer to their end-users could significantly reduce the environmental impact of aquafeeds associated to raw-material transport, but little information is available on their use in diets for this European seabass (*Dicentrarchus labrax*). Due to the current lack of knowledge on this matter, the goal of this thesis was to evaluate the potential of animal by-products as fishmeal and fish oil replacements in practical diets for European seabass, by performing the following specific objectives:

a) Characterize and select locally processed agro-food by-products as potential candidates to replace fishmeal in practical diets for seabass by assessing their nutritional value: evaluation of their chemical composition and nutrient availability.
b) Identifying the maximal fishmeal replacement level by the selected by-product by evaluating the effects of that replacement on juvenile seabass' growth performance, nutrient utilization, immune status and muscle fatty acid profile;
c) Determining the potential of different rendered animal fats as fish oil substitutes in seabass diets by evaluating their composition and their macronutrient and fatty acid digestibility;
d) Establishing the maximal level of fish oil replacement by the selected rendered fat in juvenile seabass diets through the evaluation of the effects of that replacement on growth performance, nutrient utilization, tissue fatty acid profile and lipid metabolism;
e) Using Life Cycle Assessment as a tool to assess the environmental impacts associated with the production of the by-products selected as fishmeal and fish oil replacements: identification of the impacts generated in four selected impact categories (abiotic depletion, acidification, eutrophication and global warming).

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Chapter II

Apparent digestibility coefficients of processed agro-food by-products in European seabass (*Dicentrarchus labrax*) juveniles

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Apparent digestibility coefficients of processed agro-food by-products in European seabass (*Dicentrarchus labrax*) juveniles

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Abstract

Apparent digestibility coefficients (ADCs) of processed agro-food by-products were assessed in European seabass (*Dicentrarchus labrax*). Each experimental diet was obtained by replacing 300 g kg^{-1} of a commercial-based diet used as reference (REF) with a test ingredient: wheat germ (GERM), okara meal (OKA), poultry by-product meal (POULT), steam hydrolyzed (FeHY) and enzyme-treated feather meal (FeENZ), beta-lactoglobulin (β-Lg), and peptide fractions > 3000 Da obtained from brewer's yeast (YeastP) and fish by-products (FishP). Dry matter ADC was highest in β-Lg (95%) and lowest in OKA (40%). Protein ADCs were high in β-Lg, FishP, GERM and POULT (> 94%); intermediate in FeHY and FeENZ (85 – 88%) and moderate in OKA and YeastP (76 – 78%). The essential amino acids' ADC mean was above 91% in POULT, β-Lg, GERM and FishP, 84 – 89% in FeHY and FeENZ and 73 – 76% in YeastP and OKA. Energy ADC was highest in POULT and β-Lg (89 – 95%) and lowest in YeastP and OKA (61 – 64%). Lipid ADC was highest for POULT and GERM (100%). Phosphorus ADC was lowest in GERM (19%) and highest in β-Lg (88%). FeHY, FeENZ, POULT, GERM, FishP and β-Lg are highlighted as protein sources for European seabass.

Introduction

Fishmeal is the main protein source in carnivorous fish feeds, due to its adequate amino acid profile, high palatability, for being a great source of essential fatty acids and minerals, and, until recently, for having low price and wide availability (Gatlin et al., 2007). However, the expansion of the aquaculture industry rapidly increased aquafeed production, depleting fishmeal stocks and increasing the price of this feedstuff. The economic and environmental sustainability of aquaculture depends on the identification and selection of new protein sources nutritionally adequate to each species, available in the market at a competitive price, and environment-friendly (Klinger and Naylor, 2012). Studies using life cycle assessment show that reducing fishmeal through the incorporation of locally produced products (Mungkung et al., 2013) or even fishmeal from by-products (Papatryphon et al., 2004) can decrease aquafeed environmental impact mainly through the decrease of net primary production use.

The re-approval of non-ruminant land animal meals in European aquafeeds (European Commission, 2013) has sparked the interest in searching for nutrient sources in land animal by-products, which are widely generated and often discarded, causing environmental pollution and the loss of valuable biological resources (NRC, 2011). Animal by-products can be interesting sources of protein, antioxidants and fatty acids, namely poultry by-product meal (heads, legs, bones and viscera), feather meal (Lasekan et al., 2013), and fish protein hydrolysate from fish by-products (Bougatef et al., 2010). Several vegetable by-products have also been described as excellent sources of proteins, lipids and bioactive compounds, namely wheat germ, from the flour milling industry (Brandolini and Hidalgo, 2012) and okara meal, a by-product of processing soya beans (Li et al., 2012). Spent brewer's yeast (*Saccharomyces* sp.), the brewing industry's second major by-product, has also been indicated as a potential fishmeal replacement up to 500 g kg^{-1} in feeds for European seabass (*Dicentrarchus labrax*; Oliva-Teles and Goncalves, 2001) and up to 250 g kg^{-1} in rainbow trout (*Oncorhynchus mykiss*; Rumsey et al., 1991). The low molecular weight peptide fractions obtained from agro-food by-products can have bioactive properties applicable to human health and food sectors: hydrolyzed collagen from fish by-products has anti-hypertensive properties (Ferraro et al., 2010), hydrolyzed keratin from poultry feathers has antioxidant properties (Fakhfakh et al., 2011) and spent brewer's yeast and wheat germ can have anti-hypertensive and prebiotic functions (Brandolini and Hidalgo, 2012). The technological processes used to obtain these interesting fractions for human nutrition also generate larger peptides, less valuable for humans, but potentially valuable for fish nutrition, contributing to the application of the circular economy concept.

Potential fishmeal replacers in aquafeeds should be highly digestible ingredients to improve fish performance and reduce waste production. The nutrient digestibility of an ingredient can vary from species to species due to intestinal physiology differences, so results obtained in different species are hard to compare (NRC, 2011). The by-products quality not only depends on their source, but also on the applied processing methods, which affect the bioavailability of nutrients. For instance, protein hydrolysis of poultry by-products by applying heat and pressure, enzymatically, or even by bacterial cultivation generate different mixtures of free amino acids and peptides (Fontoura et al., 2014). The duration, temperature and pH of the hydrolysis process can also affect the quality and composition of the final product (Lasekan et al., 2013). Davies et al*.* (2009) compared steam hydrolyzed with enzyme treated feather meal on European seabass and showed that the digestibility of some essential amino acids, such as histidine and methionine, varied among these two ingredients, although protein ADC (apparent digestibility coefficient) values were similar. The protein ADC value of an ingredient is the result of the apparent digestibility of each individual amino acid, but is most commonly estimated by accessing the ADC of nitrogen, which is a less expensive and faster analysis. The protein content of a feedstuff is usually estimated by multiplying its N content by a standard conversion factor (6.25) that assumes the N content of proteins to be 160 g kg^{-1}. However, this value can differ among protein sources, depending on their amino acid composition, since the N content of amino acids can vary substantially, but this is usually disregarded (Mariotti et al., 2008). The use of N content to determine protein ADC can produce inaccurate values, therefore individual amino acid digestibility should be used when assessing potential protein sources (NRC, 2011), particularly to evaluate ingredients with low protein content (Guimaraes et al., 2008; Wilson et al., 1981).

Protein ADC of some agro-food by-products has been reported in several marine fish species, ranging from 78 – 86% for poultry by-product meal in European seabass, Atlantic cod (*Gadus morhua*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*; Davies et al., 2009; Tibbetts et al., 2006), from 22 – 79% for hydrolyzed feather meal protein in gilthead seabream, turbot, Atlantic cod, European seabass and rockfish (*Sebastes schlegeli*; Davies et al., 2009; Lee, 2002; Tibbetts et al., 2006) and from 92 to 100% in wheat by-products such as wheat middling's and wheat gluten in hybrid striped bass, red drum (*Sciaenops ocellatus*) and Atlantic cod (McGoogan and Reigh, 1996; Sullivan and Reigh, 1995; Tibbetts et al., 2006). The protein ADC of brewer's yeast has also been evaluated in juvenile rockfish (73%; Lee, 2002).

European seabass is one of the most important marine fish species produced in Mediterranean countries (156,000 t produced in 2014; FAO, 2017). It is a carnivorous fish species with a high protein requirement (450 g kg^{-1}; Fournier et al., 2002) reinforcing the need for alternative protein sources for its sustainable production. Moreover, seabass production

costs are deeply associated with aquafeeds' price (> 50% of total costs; Rana et al., 2009). The search for new ingredients has therefore a double interest – the environmental sustainability of the sector and the international competitiveness of local enterprises in the global market. Although the replacement of fishmeal by vegetable sources has been successfully achieved in this species without impairing growth performance (Kaushik et al., 2004; Le Boucher et al., 2013), plant ingredients still compete with human nutrition. The inclusion of by-products produced closer to their end-users could significantly reduce the environmental impact of aquafeeds associated to raw-material transport (Mungkung et al., 2013), but little information is available on its use in diets for European seabass.

The present study was conducted to assess the *in vivo* apparent digestibility coefficients (ADCs) of distinct processed agro-food by-products to determine their potential as alternative protein sources in European seabass (*Dicentrarchus labrax*) juveniles. The selected by-products are generated in large quantities by local agro-food industries (flour milling industry, soy milk production, poultry farms, cheese and beer production and canning industries) and under a circular economy perspective, the valorization of such by-products may allow their reintroduction in the market, decreasing waste and providing added value for the industry.

Materials and methods

The present study was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

Ingredient origin and experimental diets

Table 8. Portuguese food-industry by-products, processing methods applied, and selected peptide fractions used as test ingredients.

Ingredient	Processing method	Selected fraction
Wheat germ[1]	By-product of wheat processing. Used directly as test ingredient.	Whole meal (GERM)
Okara[2]	By-product from soymilk production process. Autoclaving (1 atm, 121 ºC for 20 min) followed by drying until constant weight (65 ºC for 68 hours).	Whole okara meal (OKA)
Poultry by-product[3]	Poultry by-products (skin, viscera, bone and bruised meat). Steam cooked at 101 ºC followed by fat extraction using a screw press.	Whole poultry meal (POULT)
Feathers[4]	Poultry feathers mixed with poultry blood (about 250 g kg^{-1} blood) and subjected to steam/pressure hydrolysis (133 ºC; 304 kPa).	Whole hydrolysate feather meal (FeHY)
Feathers[4]	Poultry feathers mixed with poultry blood (about 250 g kg^{-1} blood) and incubated for 45 minutes at 45-50 ºC with the enzyme AG175™ [5] (1.5 kg enzyme and 15 L water per ton of feather-blood mix), followed by steam/pressure hydrolysis (120 ºC; 203 kPa).	Whole hydrolysate feather meal (FeENZ)
Milk whey[6]	Membrane separation with protein concentration, followed by hydrolysis by commercial cardosins and nanofiltration (3000 Da).	Beta-lactoglobulin (β-Lg)
Spent brewer's yeast[7]	Membrane separation with protein concentration, followed by hydrolysis by commercial cardosins and nanofiltration (3000 Da).	Peptide fraction > 3000 Da (YeastP)
Fish processing by-product[8]	Solid homogenization of sardine leftovers (skin and meat), followed by membrane separation with protein concentration and hydrolysis by commercial cardosins and nanofiltration (3000 Da).	Peptide fraction > 3000 Da (FishP)

[1] Germen - Moagem de Cereais, Portugal.
[2] Nutre S.A., Portugal.
[3] Savinor Sociedade Avícola do Norte, S.A., Portugal.
[4] Avicasal - Sociedade Avícola SA, Portugal.
[5] Jefo Nutrition Inc., Saint-Hyacinthe, Quebéc, Canada.
[6] Queijo Saloio - Indústria de Laticínios, S.A., Portugal.
[7] Unicer - Bebidas, S.A, Portugal.
[8] Fábrica De Conservas A Poveira S.A., Portugal.

Different by-products generated in large quantities by local agro-food industries (flour milling industry, soy milk production, poultry farms, cheese and beer production and canning industries) were either directly used as test ingredients (wheat germ) in European seabass, or after appropriate technological processing (okara meal, poultry by-product meal, steam hydrolyzed and enzyme-treated feather meal, beta-lactoglobulin from milk whey). When

processed, the resulting product was either used entirely as test ingredient or a protein fraction was selected instead, as shown in Table 8. In some cases, the smallest peptide fractions resulting from processing some by-products (spent brewer's yeast and fish by-products) were directed to human nutrition, due to their high biological value (Amorim et al., 2016), and only the highest molecular peptide fractions (> 3000 Da), considered less interesting for human nutrition, were selected as test ingredients for European seabass. In this study, selected high molecular peptide fractions (> 3000 Da) were obtained by hydrolysis with *Cynara cardunculus* enzymes from both spent brewer's yeast and processed sardine leftovers (skin and meat) from the canning industry. The proximate composition and amino acid composition of each test ingredient is presented in Table 9.

Table 9. Proximate composition and amino acid profile of the test ingredients.

Test ingredient	GERM	OKA	POULT	FeHY	FeENZ	β-Lg	YeastP	FishP
Dry matter (DM, g/kg)	885	955	973	944	959	918	930	954
Ash (g/kg DM)	46	34	137	47	36	35	59	130
Crude protein (g/kg DM)	277	269	717	855	862	794	394	859
Crude fat (g/kg DM)	115	103	131	112	99	28	12	28
Gross energy (MJ/kg DM)	21.1	21.2	21.7	24.3	24.2	23.9	19.5	20.9
Phosphorus (g/kg DM)	12	3	23	7	6	10	9	11
Crude fiber (g/kg DM)	31	180	11	8	10	2	2	1
NDF[1] (g/kg DM)	133	426	ND[2]	ND[2]	ND[2]	ND[2]	11	ND[2]
Starch (g/kg DM)	218	3	ND[2]	ND[2]	ND[2]	ND[2]	222	ND[2]
Essencial amino acids (g/kg DM)								
Arginine	24	21	38	89	77	23	14	117
Histidine	8	7	9	13	12	15	13	14
Lysine	19	16	19	38	40	86	41	34
Threonine	14	12	25	42	40	52	18	36
Isoleucine	11	12	27	36	36	48	17	22
Leucine	20	21	44	68	67	89	35	44
Valine	15	15	41	56	56	44	22	27
Methionine	6	4	9	8	8	21	9	10
Phenylalanine	12	14	32	46	42	34	18	21
Non-essencial amino acids (g/kg DM)								
Cystine	6	1	16	27	23	19	3	vest[3]
Tyrosine	9	9	17	30	30	28	20	17
Asp acid + Asparagine	27	128	36	59	59	79	23	29
Glut acid + Glutamine	51	43	57	88	89	124	84	188
Alanine	19	12	34	39	40	42	12	vest[3]
Glycine	19	12	42	60	61	18	8	76
Proline	15	14	47	75	74	48	34	vest[3]
Serine	15	15	47	97	102	39	21	219

[1] NDF, Neutral detergent fiber;
[2] ND, Not determined, in the animal ingredients;
[3] vest, vestigial amount of amino acid (< 1 g/kg DM).

A commercial-based diet for European seabass was formulated and extruded by SORGAL S.A, Portugal, and used as basal mixture (Table 10). To this mixture, 10 g kg^{-1} chromic oxide (Cr_2O_3, Merck KGaA, Germany) was added as an inert marker for the evaluation of the apparent digestibility coefficient (ADC). The reference diet (REF) consisted in 1000 g kg^{-1} of the basal mixture. Seven test diets were produced by mixing 700 g kg^{-1} of the basal

mixture and 300 g kg^{-1} of each test ingredient (GERMd – wheat germ diet; OKAd – okara meal diet; POULTd – poultry by-product meal diet; FeHYd – steam hydrolyzed feather meal diet; FeENZd – enzymatically hydrolyzed feather meal diet; β-Lgd – beta-lactoglobulin diet; YeastPd – spent brewer's yeast peptides > 3000 Da diet; FishPd – fish processing by-product meal peptides > 3000 Da diet). Formulation, chemical composition and energy content of the basal mix and experimental diets are shown in Table 10. All experimental diets were mixed and dry pelleted through a 3.2 mm die at 50 ºC using a laboratory pellet press (CPM, C-300 model, S. Francisco, USA).

Digestibility trial

The digestibility trial was conducted in the Fish Culture Experimental Unit of CIIMAR (Porto, Portugal), with juvenile European seabass (*Dicentrarchus labrax*) obtained from Maresa – Mariscos de Estero, S.A. (Ayamonte, Spain). In order to adapt to the experimental conditions, fish were kept in quarantine for 4 weeks prior to the experiment and fed with the reference diet (without chromic oxide). After acclimatization, six homogeneous groups of 20 fish (body weight 15 ± 2 g) were randomly distributed by 6 tanks of 55 L with individual feces sedimentation columns (Guelph system), specially designed according to Cho and Slinger (1979). Fish were then adapted to the new conditions for 15 days (water temperature of 20 ± 1 ºC, salinity of 35 g L^{-1}, flow rate at 2 $L.min^{-1}$ and 12 h light/12 h dark photoperiod regime).

After the conditioning period, the experimental diets were fed once a day until apparent satiation of the fish for 5 days to adapt to each experimental diet before the feces collection began. Fish continued to be fed *ad libitum*, once a day, during the period in which feces were collected. Diets were tested in triplicate with the exception of the high molecular peptide fractions and wheat germ, which were tested in duplicate due to their lower availability. About 30 minutes after feeding, every tank was carefully cleaned to assure that there would be no remains of uneaten feed left in the tanks and in the sedimentation column. Feces were collected from the sedimentation column every morning, before feeding, and then centrifuged to eliminate water excess before freezing at −20 ºC. Daily collection of the feces was performed for each experimental diet until obtaining an amount considered enough for chemical analysis (8 – 10 days). At the end of the trial, feces were freeze-dried prior to analysis. Since the recirculating water system used was only constituted by 6 tanks, this procedure had to be repeated over time with different diets. Replicates of the reference diet were tested both with the first and last ingredients. Fish were fasted for 24 h between diets, allowing the first 5 days of feeding for adaption to the new diet. The remaining procedure was performed as described above.

Table 10. Formulation and proximate composition of the basal mixture and experimental diets.

Ingredients (g kg^{-1})	
Fishmeal 60% CP [1]	282
Poultry meal [2]	250
Fish oil [3]	140
Maize (OGM free) [4]	109
Sunflower seed meal (dehulled) [5]	71
Maize gluten [6]	50
Pea starch [7]	30
Fishmeal 68% CP [8]	25
Haemoglobin [9]	20
Vitamin and Mineral premix [10]	8
L-Lysine [11]	6
Binder [12]	5
Hylises™ [13]	3
DL-Methionine [14]	1

	Experimental diets								
	REF	GERMd	OKAd	POULTd	FeHYd	FeENZd	β-Lgd	YeastPd	FishPd
Basal mix (g kg^{-1})	1000	700	700	700	700	700	700	700	700
GERM (g kg^{-1})		300							
OKA (g kg^{-1})			300						
POULT (g kg^{-1})				300					
FeHY (g kg^{-1})					300				
FeENZ (g kg^{-1})						300			
β-Lg (g kg^{-1})							300		
YeastP (g kg^{-1})								300	
FishP (g kg^{-1})									300
Proximate composition									
Dry matter (DM, g kg^{-1})	953	936	946	946	956	961	946	948	954
Ash (g kg^{-1} DM)	111	91	105	133	90	88	88	94	113
Crude protein (g kg^{-1} DM)	467	436	412	545	582	579	555	445	580
Crude fat (g kg^{-1} DM)	241	210	208	210	206	206	189	185	188
Gross energy (kJ g^{-1} DM)	23.4	22.8	23.2	23.4	23.5	23.6	23.7	22.4	22.8
Phosphorus (g kg^{-1} DM)	14	10	12	18	10	9	10	11	12

The abbreviations for the experimental diets stand for: REF – reference diet; GERMd – wheat germ diet; OKAd – okara meal diet; POULTd – poultry by-product meal diet; FeHYd – steam hydrolyzed feather meal diet; FeENZd – enzyme hydrolyzed feather meal diet β-Lgd – beta-lactoglobulin diet; YeastPd – spent brewer's yeast peptides > 3000 Da diet; FishPd – fish processing by-product meal peptides > 3000 Da diet.

[1] Byproduct fishmeal: 600 g kg^{-1} crude protein (CP), 120 g kg^{-1} crude fat (CF), Savinor S.A., Portugal.

[2] Byproduct poultry meal: 670 g kg^{-1} CP, 140 g kg^{-1} CF, Savinor S.A., Portugal.

[3] Savinor S.A., Portugal.

[4] Oleocom S.A. Portugal.

[5] 340 g kg^{-1} CP, 190 g kg^{-1} crude fibre, Sotrapex Lda., Portugal.

[6] 580 g kg^{-1} CP, Copam S.A., Portugal.

[7] ESASA S.A., Spain.

[8] Peruvian fishmeal Super Prime: 680 g kg^{-1} CP, 90 g kg^{-1} CF, Sopropêche, France.

[9] AP 301®, APC, Spain.

[10] Vitamins (IU or mg/kg diet): vitamin A, 5.000 IU ; vitamin D3, 1.000 IU ; vitamin E/all-racemic alpha tocopheryl acetate, 250 IU ; vitamin C (ascorbyl monophosphate), 100.0 mg ; sodium menadione bisulphate, 6.0 mg; thiamine, 4.8 mg; riboflavin, 12.0 mg; niacin, 18.0 mg; calcium panthotenate, 42.0 mg; pyridoxine, 6.0 mg; folic acid, 3.6 mg; vitamin B12, 0.012 mg; inositol, 180 mg; biotin, 0.42 mg; choline chloride, 375 mg, betaine, 500 mg. Minerals (g or mg/kg diet): iron sulphate heptahydrate, 37.5 mg; hydrated ferrous amino acid chelate, 50.0 mg; potassium iodide, 1.0 mg; cobalt sulphate heptahydrate, 0.06 mg; copper sulphate pentahydrate, 3.2 mg; hydrated copper amino acid chelate 2.0 mg; manganese oxide, 12.5 mg; hydrated manganese amino acid chelate, 8.0 mg; zinc (hydrated zinc amino acid chelate) 50.0 mg; zinc oxide, 12.5 mg; sodium selenite, 0.20 mg; organic selenium from *Saccharomyces cerevisae*, 0.20 mg. Antioxidants (mg/kg diet): propyl gallate, 40.0 mg; BHA, 40.0 mg.

[11] ADM - Archer Daniels Midland Company, USA. Supplied by Sorgal S.A., Portugal.
[12] Kiezelguhr, Cia. Española de Kiezelguhr, S.L., Spain.
[13] ICC Lda., Brazil.
[14] Evonik Industries A.G., Germany.

The apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Maynard et al. (1979): ADC (%) = 100 × (1 − (dietary Cr_2O_3 level/feces Cr_2O_3 level) × (feces nutrient or energy level/dietary nutrient or energy level). ADC of dry matter was calculated as follow: ADC (%) = 100 × (1 − (dietary Cr_2O_3 level/feces Cr_2O_3 level). The ADCs of nutrients and energy of the test ingredients were estimated according to NRC (2011): ADC_{ing} (%) = ADC_{test} + [(ADC_{test} − ADC_{ref}) × ((0.7 × D_{ref}) / (0.3 × D_{ing}))]; where ADC_{test} = ADC (%) of the experimental diet, ADC_{ref} = ADC (%) of the reference diet, D_{ref} = g kg^{-1} nutrient (or kJ kg^{-1} gross energy) of the reference diet (DM basis); D_{ing} = g kg^{-1} nutrient (or kJ kg^{-1} gross energy) of the test ingredient (DM basis).

Chemical analysis

Every test ingredient, experimental diet and collected feces were ground (feces were sifted) and homogenized before analysis. Proximate composition analysis was performed in duplicate and according to the Association of Official Analytical Chemists (AOAC) (2006) methods. All samples were analyzed for dry matter (105 ºC for 24 h); ash by combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 500 ºC for 5 h); crude protein (N × 6.25, except for Beta-lactoglobulin (N × 6.32) and wheat germ (N × 4.99), as suggested by Mariotti et al. (2008)) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA); crude fat content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany); gross energy was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany); and phosphorus content by digestion at 230 ºC in a Kjeldatherm block digestion unit followed by digestion at 60 ºC in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406). Ingredients were analyzed regarding their crude fiber content according to the intermediate filtration method (ISO 6865:2000); neutral detergent fiber (NDF) was determined according to ISO 16472:2006 (Robertson and Van Soest, 1981; Van Soest and Robertson, 1985); starch was determined as described in Thivend et al. (1972). Chromic oxide content in diets and feces was determined according to Bolin et al. (1952).

Amino acid analysis

Samples from test ingredients, experimental diets and collected feces were hydrolyzed (6 M HCl at 116 ºC over 24 h in nitrogen-flushed glass vials) before total amino acid analyses. All samples were then pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Tryptophan was not determined, since it is partially destroyed by acid hydrolysis. The resultant peaks were analyzed with EMPOWER software (Waters, USA).

Statistical analysis

ADCs data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and transformed whenever required before being submitted to a one-way ANOVA with the statistical program IBM SPSS STATISTICS, 22.0 package, IBM corporation, New York, USA (2011). When this test showed significance, individual means were compared using HSD Tukey Test. In all cases significant differences were considered when $p < 0.05$.

Results

The proximate composition of the test ingredients is portrayed in Table 9. Crude protein content of the selected ingredients ranged from 277 to 862 g kg^{-1}, crude fat varied between 12 and 131 g kg^{-1} and gross energy from 19 to 24 kJ g^{-1}. Phosphorus content of the test ingredients ranged from 3 to 23 g kg^{-1}. Crude fiber varied from 1 to 180 g kg^{-1} among test ingredients and the starch content ranged from 3 to 222 g kg^{-1}. Neutral detergent fiber was only analyzed on the ingredients with cellular walls and ranged from 11 to 426 g kg^{-1}. The amino acid profile of the test ingredients is also presented in Table 9. Threonine, phenylalanine and tyrosine are present in most ingredients in similar proportions, but most amino acids (either essential or non-essential) exist in very different proportions among test ingredients, except in the case of the two hydrolyzed feather meals, which were quite similar. The 300 g kg^{-1} inclusion of these test ingredients resulted in experimental diets that had 412 to 582 g kg^{-1} crude protein, 185 to 241 g kg^{-1} crude fat, 22 to 24 kJ g^{-1} gross energy and 9 to 18 g kg^{-1} phosphorus, reflecting the variability in the composition of each test ingredient (Table 10).

No mortality was observed during the trial. The apparent digestibility coefficients (ADCs) of the experimental diets and feed ingredients are reported in Table 11. In the experimental diets, ADC of the dry matter varied from 67 to 83%, with OKAd displaying a significantly lower value compared to REF, whereas β-Lgd had the highest ADC. Digestibility of protein was high in all diets (87 – 95%), but fish fed β-Lgd and FishPd showed higher digestibility values ($p < 0.05$) than those fed REF. Contrarily, fish fed YeastPd and OKAd registered lower protein digestibility than the ones fed REF. Energy digestibility varied from 80 to 89%; the lowest values ($p < 0.05$) were recorded in fish fed the diets YeastPd and OKAd. ADC of lipids was high in every group (> 91%), and fish fed YeastPd had lower values than those fed REF ($p < 0.05$). Phosphorus digestibility ranged from 48 to 68%, being lower in fish fed the diets GERMd and POULTd than in fish fed REF.

Table 11. Apparent digestibility coefficients (ADC) of nutrients and energy of experimental diets and feed ingredients.

ADC Diets (%)	REF	GERMd	OKAd	POULTd	FeHYd	FeENZd	β-Lgd	YeastPd	FishPd
Dry matter	78.5 ± 1.5^{bc}	78.2 ± 0.4^{bc}	66.9 ± 1.8^{e}	75.8 ± 0.4^{cd}	76.9 ± 1.0^{bc}	80.1 ± 1.3^{ab}	83.3 ± 0.1^{a}	72.3 ± 1.7^{d}	80.5 ± 0.9^{ab}
Protein	91.6 ± 1.3^{cd}	92.7 ± 0.0^{bc}	87.3 ± 0.6^{e}	92.3 ± 0.1^{bc}	88.5 ± 1.1^{e}	89.8 ± 0.2^{de}	94.8 ± 0.3^{a}	87.9 ± 1.1^{e}	94.1 ± 0.4^{ab}
Energy	86.5 ± 1.1^{bc}	87.1 ± 0.5^{ab}	80.1 ± 1.2^{d}	87.3 ± 0.5^{ab}	84.4 ± 0.9^{c}	85.9 ± 0.3^{bc}	89.0 ± 0.2^{a}	79.9 ± 1.2^{d}	86.9 ± 0.7^{ab}
Lipids	95.1 ± 0.0^{abc}	95.8 ± 0.2^{ab}	93.9 ± 1.2^{c}	96.0 ± 0.2^{a}	93.4 ± 0.8^{cd}	94.1 ± 0.5^{bc}	95.23 ± 0.0^{abc}	91.4 ± 0.6^{d}	94.8 ± 0.0^{abc}
Phosphorus	61.8 ± 2.0^{ab}	50.2 ± 2.4^{cd}	60.0 ± 1.0^{b}	47.8 ± 2.2^{d}	55.2 ± 0.8^{bc}	59.7 ± 2.1^{b}	68.2 ± 1.5^{a}	57.1 ± 2.3^{bc}	61.6 ± 3.0^{ab}

ADC Ingredients (%)	GERM	OKA	POULT	FeHY	FeENZ	β-Lg	YeastP	FishP
Dry matter	77.4 ± 1.5^{bc}	39.8 ± 5.8^{d}	69.5 ± 1.4^{bc}	73.1 ± 3.4^{bc}	83.8 ± 4.4^{b}	95.0 ± 0.2^{a}	57.6 ± 5.6^{c}	86.8 ± 2.9^{b}
Protein	97.2 ± 0.2^{a}	70.0 ± 2.8^{d}	93.4 ± 0.2^{ab}	84.6 ± 2.6^{bc}	87.6 ± 0.4^{b}	99.2 ± 0.6^{a}	77.8 ± 4.0^{cd}	97.2 ± 0.9^{a}
Energy	88.7 ± 1.6^{ab}	63.6 ± 4.3^{c}	89.5 ± 1.9^{ab}	79.6 ± 3.0^{b}	84.8 ± 0.9^{b}	94.9 ± 0.5^{a}	61.4 ± 4.6^{c}	88.0 ± 2.6^{ab}
Lipids	99.6 ± 0.9^{a}	87.4 ± 7.6^{a}	99.7 ± 1.2^{a}	85.0 ± 5.0^{a}	88.0 ± 4.2^{a}	98.9 ± 0.8^{a}	-19.1 ± 17.2^{b}	89.7 ± 1.0^{a}
Phosphorus	18.9 ± 9.0^{c}	43.3 ± 10.9^{bc}	29.5 ± 4.3^{bc}	23.4 ± 4.8^{bc}	49.1 ± 12.5^{bc}	88.4 ± 6.3^{a}	39.4 ± 10.7^{bc}	61.0 ± 12.0^{ab}
DE (kJ g^{-1})	18.7 ± 0.4^{b}	13.5 ± 0.9^{c}	19.4 ± 0.4^{b}	19.4 ± 0.8^{b}	20.4 ± 0.4^{b}	22.7 ± 0.1^{a}	12.0 ± 0.9^{c}	18.4 ± 0.5^{b}

Values are presented as mean ± standard deviation (n = 2, n = 3 (REF, POULT, FeHY and FeENZ)). Values in the same row without a common superscript letter differ significantly ($p < 0.05$).
Absence of superscript indicates no significant difference between treatments.
DE - digestible energy of the test ingredients, calculated as ADC_{energy} × gross energy (kJ g^{-1} DM).

Table 12. Apparent digestibility coefficients (ADC) of individual amino acids in the test ingredients.

ADC (%)	GERM	OKA	POULT	FeHY	FeENZ	β-Lg	YeastP	FishP
Essential amino acids								
Arg	94.7 ± 0.7^{ab}	79.3 ± 2.0^{bc}	97.0 ± 0.1^{ab}	90.2 ± 0.7^{ab}	90.7 ± 0.1^{ab}	106.6 ± 0.7^{a}	57.7 ± 9.0^{c}	98.1 ± 0.3^{ab}
His	95.0 ± 1.1^{abc}	92.2 ± 1.7^{d}	93.9 ± 2.1^{abc}	86.8 ± 0.9^{bc}	93.5 ± 0.1^{abc}	100.0 ± 0.7^{a}	84.9 ± 3.9^{cd}	97.6 ± 1.1^{ab}
Lys	88.5 ± 1.5^{cd}	81.7 ± 1.4^{e}	92.9 ± 2.4^{bc}	88.1 ± 0.4^{cd}	89.9 ± 0.1^{cd}	98.1 ± 0.4^{ab}	92.4 ± 2.8^{d}	99.4 ± 0.0^{a}
Thr	96.8 ± 3.0^{a}	71.4 ± 2.8^{b}	95.7 ± 0.8^{a}	79.8 ± 0.7^{ab}	84.9 ± 0.1^{ab}	97.2 ± 0.5^{a}	66.7 ± 6.2^{b}	97.9 ± 0.9^{a}
Iso	97.0 ± 4.1^{a}	74.4 ± 2.7^{bc}	89.7 ± 0.4^{ab}	79.7 ± 0.7^{abc}	84.3 ± 0.1^{ab}	93.2 ± 0.7^{a}	66.3 ± 4.8^{c}	95.0 ± 2.0^{a}
Leu	93.9 ± 3.3^{ab}	76.1 ± 2.0^{d}	93.1 ± 1.4^{ab}	81.0 ± 0.7^{bcd}	84.6 ± 0.0^{bc}	97.3 ± 0.7^{a}	75.5 ± 4.2^{cd}	97.2 ± 1.2^{a}
Val	88.3 ± 3.6^{ab}	66.6 ± 3.2^{c}	89.3 ± 0.3^{ab}	76.3 ± 1.2^{bc}	79.7 ± 0.1^{abc}	94.7 ± 1.1^{ab}	62.0 ± 7.6^{c}	96.8 ± 2.3^{a}
Met	85.9 ± 1.8^{cde}	80.2 ± 1.4^{e}	83.1 ± 0.4^{de}	94.1 ± 0.7^{bc}	102.7 ± 0.2^{a}	99.9 ± 0.3^{ab}	86.3 ± 4.0^{cde}	90.0 ± 0.5^{cd}
Phe	95.0 ± 1.5^{ab}	74.8 ± 2.1^{c}	91.1 ± 1.4^{ab}	81.1 ± 0.6^{bc}	86.8 ± 0.1^{abc}	97.1 ± 0.1^{a}	75.0 ± 4.6^{c}	97.2 ± 1.0^{a}
EAA[1] mean	97.9 ± 1.6	76.3 ± 2.1	91.8 ± 0.0	84.1 ± 0.7	88.6 ± 0.0	98.2 ± 0.6	73.0 ± 4.3	96.6 ± 1.0
Non-essential amino acids								
Cys	93.6 ± 0.3^{a}	69.8 ± 0.2^{c}	79.8 ± 0.9^{bc}	84.1 ± 1.2^{ab}	86.0 ± 0.3^{ab}	88.6 ± 1.0^{ab}	70.2 ± 7.9^{c}	ND[2]
Tyr	91.5 ± 2.6^{abc}	69.2 ± 2.4^{e}	93.7 ± 1.8^{abc}	84.8 ± 0.5^{cd}	88.4 ± 0.2^{bcd}	98.7 ± 0.3^{ab}	75.8 ± 2.2^{de}	100.3 ± 1.5^{a}
Asp + Asn	97.8 ± 3.2^{a}	75.2 ± 2.0^{bc}	93.4 ± 1.3^{ab}	84.2 ± 0.6^{abc}	89.5 ± 0.0^{ab}	98.4 ± 1.0^{a}	67.1 ± 7.2^{c}	100.1 ± 2.4^{a}
Glu + Gln	100.4 ± 1.2^{a}	82.9 ± 0.5^{e}	95.0 ± 1.4^{abc}	87.6 ± 0.5^{cde}	91.8 ± 0.1^{bcd}	99.9 ± 0.5^{a}	85.9 ± 2.6^{de}	97.4 ± 0.7^{ab}
Ala	93.6 ± 0.5^{b}	74.8 ± 2.5^{b}	94.1 ± 0.1^{b}	84.1 ± 0.7^{b}	88.1 ± 0.1^{b}	99.8 ± 0.5^{b}	63.6 ± 6.8^{b}	ND[2]
Gly	97.1 ± 0.4^{ab}	71.3 ± 2.7^{bc}	92.9 ± 0.1^{ab}	86.4 ± 0.6^{b}	89.5 ± 0.1^{b}	107.5 ± 0.9^{a}	52.0 ± 9.2^{c}	96.3 ± 1.0^{ab}
Pro	98.7 ± 0.2^{b}	75.4 ± 0.3^{b}	91.9 ± 1.3^{b}	81.3 ± 0.6^{b}	82.4 ± 0.1^{b}	99.5 ± 0.5^{b}	82.0 ± 1.8^{b}	ND[2]
Ser	110.4 ± 0.0^{a}	85.2 ± 2.2^{c}	90.4 ± 0.4^{bc}	61.2 ± 1.0^{e}	73.6 ± 1.4^{d}	98.3 ± 1.3^{b}	85.4 ± 0.7^{c}	90.7 ± 0.0^{bc}
NEAA[3] mean	97.9 ± 1.0	75.5 ± 1.5	91.4 ± 0.9	81.7 ± 0.7	86.2 ± 0.1	98.8 ± 0.7	72.7 ± 4.8	96.9 ± 1.1

Values are presented as mean ± standard deviation (n = 2, n = 3 (POULT, FeHY and FeENZ)). Values in the same row without a common superscript letter differ significantly ($p < 0.05$).

[1] EAA, essential amino acids;
[2] ND = not determined, when the amount of amino acid in the ingredient was vestigial, the ADC could not be determined;
[3] NEAA, non-essential amino acids.

Concerning the test ingredients, ADC for dry matter varied significantly (40 – 95%) and okara meal (OKA) had the lowest value ($p < 0.05$). The highest protein digestibility values (97 – 99%) were obtained in beta-lactoglobulin (β-Lg) followed by fish processing by-product meal (FishP), and wheat germ (GERM). Poultry by-product meal (POULT – 94%), enzyme-treated feather meal (FeENZ – 88%) and steam hydrolyzed feather meal (FeHY – 85%) showed intermediate results, while spent brewer's yeast peptides (YeastP – 78%) and OKA (76%) showed the lowest values for protein ADC. Energy digestibility was lowest in YeastP and OKA (61 and 64%, respectively) and highest in β-Lg (95%). Digestible energy of the test ingredients varied from 12.0 kJ g^{-1} in YeastP to 22.7 kJ g^{-1} in β-Lg. ADC for lipids was negative in YeastP (−19%) and the highest values were obtained in POULT and GERM (100%). Finally, the lowest phosphorus digestibility value was obtained in GERM (19%) and the highest value was obtained in β-Lg (88%).

The apparent digestibility coefficients of individual amino acids are shown in Table 12. Amino acid ADCs varied a lot among test ingredients, with values above 80% for histidine, lysine and methionine in all test ingredients. Arginine was also well digested in most test ingredients except in OKA (79%) and particularly in YeastP (58%). The amino acid ADC values obtained for FeENZ were generally higher than those of FeHY, with methionine and serine being significantly better digested in the FeENZ.

Discussion

The apparent digestibility coefficient (ADC) of an ingredient reflects the capability of a certain species to utilize its nutrients, predicting its potential as a feedstuff. The variability in the composition of the processed local agro-food by-products characterized in this study reflects their origin and the processing techniques applied. Wheat germ (GERM), with the lowest dry matter and high crude fat content should be prone to oxidation. The inorganic matter (ash) was low in most ingredients, except for the fish processing by-product peptides (FishP) and the poultry by-product meal (POULT). These results were expected, since these animal by-products include bones, rich in minerals. The lowest fat contents were observed in the ingredients with highest degree of processing and fractioning (β-Lg [beta-lactoglobulin], FishP and YeastP [peptide fraction > 3000 Da obtained from spent brewer's yeast]), while the un-fractioned ingredients reported higher amounts – whey and spent yeast had low amounts of fat before being processed, while fish by-products were centrifuged to remove their lipids before being hydrolyzed. FeHY (steam hydrolyzed feather meal), FeENZ (enzyme-treated feather meal), β-Lg and FishP reported the highest amounts of crude protein and the lowest carbohydrate content, measured as crude fiber, neutral detergent fiber and starch. This indicates that these are protein rich ingredients and possible alternatives to fishmeal in aquafeeds. Contrarily, GERM, OKA (okara meal) and YeastP had the lowest protein and highest carbohydrate contents, which may compromise their dietary inclusion as protein sources for carnivorous fish species. The amino acid contents of the whole test ingredients were similar to the values previously reported in the literature (Allan et al., 2000; Bandegan et al., 2010; Guimaraes et al., 2008; NRC, 2011). Compared to a reference anchovy fishmeal (5-01-985; NRC, 2011), which is considered to have the best amino acid profile for fish nutrition, test ingredients were short in some essential amino acids and rich in others. β-Lg and YeastP are short in arginine (30 – 35 *vs* 56 g kg^{-1}) but rich in lysine (103 – 108 *vs* 80 g kg^{-1}); FeHY and FeENZ are short in histidine and lysine (14 – 15 *vs* 24 g kg^{-1}, and 47 – 44 *vs* 78 g kg^{-1}, respectively) but rich in arginine (91 – 104 *vs* 56 g kg^{-1}); POULT is short in lysine (36 *vs* 78 g kg^{-1}); OKA is deficient in lysine (65 *vs* 78 g kg^{-1}) and rich in arginine (76 *vs* 56 g kg^{-1}); GERM is deficient in lysine (68 *vs* 78 g kg^{-1}) and rich in arginine (87 *vs* 56 g kg^{-1}). FishP is rich in arginine (136 *vs* 56 g kg^{-1}) and short in all other essential amino acids. Cystine, alanine and proline (non-essential amino acids) were reported as vestigial in this ingredient (< 3 *vs* 8, 62 and 37 g kg^{-1}, respectively). This difference probably reflects the enzymatic hydrolysis applied to the fish residues and posterior selection of the highest molecular weight fraction, since arginine is an amino acid with high molecular weight, while cystine and proline have low molecular weights (Emery, 2005). Furthermore, most ingredients are low in methionine but the shortage of this amino acid might be compensated by the cystine present in the ingredient,

except in FishP, which is short in methionine and cystine. The amino acid profile of both feather meals was mostly similar, suggesting it was not affected by the different treatments, except for arginine, which was higher in FeENZ than in FeHY. This suggests that this amino acid was degraded by the higher temperature and pressure applied during hydrolysis, as suggested by Murray et al. (1965). The amino acid profile of YeastP was compared to whole yeast, since no similar fractions were described in the literature, and results were similar (NRC, 2011).

Seabass ADCs were high for most test ingredients tested. Dry matter ADC was moderate to high in all animal ingredients and in GERM, but lower for OKA and YeastP. In juvenile rockfish, Lee (2002) reported higher dry matter ADC values for steam hydrolyzed feather meal (87%) compared to this study (73%). This difference can be due to interspecies physiological differences in the digestive tract or to the ingredient composition. The dry matter ADC of an ingredient reflects the digestible portions of both its organic and inorganic matter, and the ADC of inorganic matter can be highly variable according to its mineral composition (NRC, 2011). The feather meal presently tested contained higher inorganic matter content (47 g kg^{-1} ash *vs* 30 g kg^{-1}) than that used in Lee (2002) and its phosphorus ADC was low (23%), which may have contributed to the lower dry matter ADC.

Values obtained for protein digestibility ranged from 76 to 99%. McGoogan and Reigh (1996) reported that, in red drum, protein digestibility was highest in protein rich ingredients and lowest in those rich in fiber. Accordingly, the lowest protein ADC was obtained in OKA, the ingredient with the highest fiber content and low protein content. To our best knowledge, the protein digestibility of okara meal has not been previously reported in fish, but resulted in a protein ADC (76%) below previously reported values for soybean meal in European seabass (90%; Gomes da Silva and Oliva-Teles, 1998). In sheep, protein ADC of okara (85%) was slightly higher than protein ADC of wheat hay, 72%, and starchy pellets, 80% (Eliyahu et al., 2015). Seabass is a carnivorous fish species that poorly digests okara, but its evaluation in omnivorous species such as Nile tilapia merits further research – high protein ADC (80%) in wheat has been reported by Sklan et al. (2004) suggesting this species' ability for using plant protein sources. The protein ADC in YeastP (78%) was higher than the one reported for whole brewer's yeast by Lee (2002) in juvenile rockfish (73%). In European seabass, protein ADC of brewer's yeast had already been reported as being poorly digested (53%) compared to fishmeal (91%; Metailler and Huelvan, 1993). A 50% replacement of fishmeal by brewer's yeast in European seabass led to a decrease in protein ADC from 93 to of 87% (Oliva-Teles and Goncalves, 2001). In the present study, a peptide fraction (> 3000 Da) selected after hydrolyzing spent brewer's yeast was tested instead of the whole yeast. This fraction also contained the beta-glucans present in yeast composition, which have previously shown to increase the feed digestibility in calves, at an inclusion level up to 75 mg kg^{-1} (Ma et al., 2015).

It is known that peptides obtained from protein hydrolysis are better digested than native proteins (Carvalho et al., 2004). Nevertheless, the largest peptides fraction can be harder to digest than native proteins, since the removal of small peptides from protein hydrolysate may lead to reduced protein ADC, fish growth and feed efficiency (Aksnes et al., 2006). In this study, however, the fraction used seems a better protein source for European seabass than the whole brewer's yeast previously tested by Metailler and Huelvan (1993).

The protein ADCs obtained for feather meal (steam hydrolyzed or enzyme-treated) were higher (85 – 88%) than values reported for European seabass (61 – 67%; Davies et al., 2009) and other saltwater fish species (22 – 83%; Booth et al., 2013; Chi et al., 2017; Davies et al., 2009; Glencross, 2011; Hernandez et al., 2014; Safari et al., 2016). Similar values have been reported in salmonids and in Japanese seabass, *Lateolabrax japonicus* (86%; Sugiura et al., 1998; Wang et al., 2012). The feather meals presently tested had a higher protein content (85 – 86%) than the ones previously tested in European seabass (77 – 82%). Several studies have related higher protein contents to an increase in protein digestibility (Austreng and Refstie, 1979; Santinha et al., 1996). Moreover, the inclusion of poultry blood (at about 250 g kg^{-1}) in the feather meal mixture may have contributed to the high protein ADCs observed in the present study – the protein ADC value of blood meal has been reported in European seabass as 91% (Gomes da Silva and Oliva-Teles, 1998). Furthermore, the results obtained can be a consequence of the hydrolysis methodology applied to the feather meal, namely the duration, pressure and temperature of the steam hydrolysis procedure, and the type of enzyme used in the enzymatic hydrolysis procedure, which may have made the peptides more available for fish. Although not statistically significant, FeENZ had a slightly higher protein ADC value than FeHY, confirming previous results by Davies et al. (2009) in European seabass.

GERM, the ingredient with the lowest protein content, had a very high protein digestibility. Data on wheat germ digestibility in fish is scarce, but Zhou et al. (2008) reported a protein digestibility of 90% in bluntnose black bream, in which the ingredient used had a higher protein content (377 g kg^{-1}), but also a higher ash content (70 g kg^{-1} *vs* 46 g kg^{-1} in the presently tested ingredient). This higher inorganic matter content may have impaired the digestibility of the ingredient, through interactions among nutrients' ADCs (NRC, 2011). This important by-product of the flour milling industry merits further evaluation as a potential protein source in aquafeeds, since European seabass digests it extremely well. Nevertheless, its high water content (> 100 g kg^{-1}) might compromise its storage and further utilization as is, suggesting that further drying before incorporation in aquafeeds is advisable.

Protein digestibility of POULT (94%) was higher than values reported in the same species by Davies et al. (86%; 2009) and in other saltwater fish species (40 – 93%; Booth et al., 2013; Chi et al., 2017; Davies et al., 2009; Glencross, 2011; Glencross et al., 2017; Nengas et al., 1995; Safari et al., 2016; Tibbetts et al., 2006; Wang et al., 2012; Wei et al., 2015). However, similar and even higher values have been reported in salmonids (94 – 96%; Sugiura et al., 1998). This difference could result from the rendering process or the source and quality of the poultry by-products used. Nevertheless, this seems a very promising protein source to incorporate in European seabass diets.

Protein ADCs of β-Lg and FishP (> 97%) were higher than values reported for fishmeal in European seabass (90 – 96%; Davies et al., 2009; Gomes da Silva and Oliva-Teles, 1998). These ingredients were obtained from by-products massively generated by local industries, and while a hydrolysis process is associated with the obtainment and selection of the fractions used, with an associated cost, the hydrolysis process applied apparently led to a high availability of the nutrients in these ingredients. β-Lg has been reported to become more easily digested after hydrolysis, even though in its native form this protein is considered to be an allergen and therefore less susceptible to digestive enzymes (Reddy et al., 1988).

The ADCs of individual amino acids varied among test ingredients and were coherent with the values estimated for crude protein ADC, with the mean ADC of essential amino acids being highest in β-Lg, GERM, FishP and POULT (> 91%). ADC values above 100% were recorded in GERM (serine, 110%), FeENZ (methionine, 103%) and β-Lg (arginine, 107%, and glycine, 108%). Values above 100% have been previously reported in different ingredients by other authors (Allan et al., 2000); this overestimation may be caused by minor analytical errors or interactions between nutrients in the reference diet and in the test ingredient (NRC, 2011), when the content of a certain amino acid in the test ingredient is very low. The low content of a specific amino acid in an ingredient (that comprised 300 g kg^{-1} of each experimental diet) can lead to a higher uptake of that amino acid from the reference diet components (that comprises 700 g kg^{-1} of the diet), in order to reach the required levels by the species. If the uptake of that amino acid is higher in that portion than the value achieved in the reference diet treatment, it will lead to an estimated amino acid ADC value above 100% in the ingredient. Amino acids ADCs for both FeHY and FeENZ were higher than the ones reported by Davies et al. (2009) for the same fish species. Furthermore, and contradicting that paper, in the present study FeENZ reported higher digestibility values for all amino acids than FeHY. However, this was only statistically significant for methionine and serine, suggesting that these amino acids became less available with the steam/pressure treatment applied to the feather meal than with the enzyme hydrolysis. In FishP, the levels of cystine, alanine and proline were vestigial so the ADCs of these amino acids could not be determined.

The high protein digestibility of β-Lg, FishP, GERM and POULT (> 94%) suggests that these are protein sources well accepted by European seabass and may replace fishmeal in feeds for this species without decreasing (and even increasing) the protein digestibility of the diets. Furthermore, the ADC of essential amino acids exceeded 91% in these ingredients, supporting the potential of these ingredients as amino acid sources for European seabass. FeHY and FeENZ had intermediate protein (85 – 88%) and essential amino acid (84 – 89%) ADCs, showing potential as protein feedstuffs for this species. OKA and YeastP had considerably lower protein ADC values (76 – 78%), so their further utilization in seabass diets has to be evaluated with caution.

Lipid digestibility was high for all test ingredients except YeastP, which showed a negative lipid ADC value. Lipid digestibility is highly dependent on the fatty acid composition and structure of the lipid source, being impaired when it is rich in saturated fatty acids (Hua and Bureau, 2009). Although the fatty acid profile of the *Saccharomyces cerevisiae* peptide fraction was not evaluated, this species has high contents of saturated fatty acids (Blagovic et al., 2001). This low ADC can also be due to interactions between ingredients, since the reference diet has a much higher lipid content (241 g kg^{-1}) than this ingredient (12 g kg^{-1}), which could have impaired the measurement of this ingredient's lipid ADC (NRC, 2011). No previous studies were found regarding lipid ADC of brewer's yeast in fish to compare to the obtained results. Lipid digestibility was high for the remaining ingredients, with the highest value in POULT and GERM (100%) and lowest in FeHY (85%). Lipid ADCs obtained for β-Lg, GERM and POULT (99 – 100%) exceeded reported values for fishmeal in European seabass (93%; Davies et al., 2009), suggesting these could be interesting alternatives to fishmeal as lipid sources, depending on their fatty acid profile. For the remaining ingredients, the values reported were lower (−19 – 90%) than for fishmeal.

The digestible energy of an ingredient, and not only its digestible protein, affects fish performance, since it is the fractional sum of the protein, lipids and carbohydrates ADCs of an ingredient (NRC, 2011). Energy digestibility of the test ingredients reflected the digestibility of their nutrients, being highest for the ingredients from animal sources and GERM, and lowest for OKA and YeastP. Energy ADCs of FeHY (80%) and FeENZ (85%) exceeded the values reported by Davies et al. (2009) for both steam and enzymatically hydrolyzed feather meal in European seabass (58 and 61%, respectively). For the remaining animal by-products and GERM, energy ADCs were high (88 – 95%) and in the range of data reported for fishmeal in European seabass (86 – 95%; Davies et al., 2009; Gomes da Silva and Oliva-Teles, 1998). Energy digestibility was lowest for YeastP and OKA (61 – 64%), agreeing with the reported negative relation between energy digestibility and fiber content of an ingredient (McGoogan and Reigh, 1996).

Phosphorus digestibility reflects the amount of this mineral absorbed by fish, indicating its contribution to water pollution, and it ranged from 19 to 88%. The lowest value was obtained in GERM (19%); the main phosphorus form present in seeds is phytate phosphorus, highly indigestible for fish (Cheng and Hardy, 2002). The addition of microbial phytase may help hydrolyze phosphorus and increase its availability. Unfortunately, at this time there are no such phytases approved for seabass feeds in the European Union.

The present results show that FeHY, FeENZ, POULT, GERM, FishP and β-Lg are well digested by European seabass (protein ADC > 85%). These ingredients showed high protein and energy digestibility and could replace fishmeal without impairing the diets digestibility. Moreover, as these by-products are produced in large quantities in Europe they can reduce the need for imported feedstuffs to include in seabass diets, reducing the carbon footprint associated with transportation. The impact of such ingredients in European seabass diets warrants further evaluation in order to determine its maximal inclusion level to assure good growth performance and nutritional quality of fish. Moreover, the final quality, availability and price of any processed by-product must be considered when considering its use in feed formulation.

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Chapter III

Hydrolyzed feather meal as a partial fishmeal replacement in diets for European seabass (*Dicentrarchus labrax*) juveniles

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Hydrolyzed feather meal as a partial fishmeal replacement in diets for European seabass (*Dicentrarchus labrax*) juveniles

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Abstract

The effects of replacing fishmeal by increasing levels of hydrolyzed feather meal (HF) on the nutrient utilization, growth performance and muscle fatty acid composition of European seabass (*Dicentrarchus labrax*) juveniles were evaluated. The humoral non-specific immune parameters were also evaluated in plasma. A growth trial was conducted for 18 weeks, using a practical feed formulation with 32% fishmeal and no HF added, as control (FM). Three experimental diets were formulated with the inclusion of 5 (HF5), 7.5 (HF7.5) and 12.5% HF (HF12.5), leading to a replacement of about 28, 55 and 76% fishmeal compared to the FM diet.

The protein apparent digestibility coefficient (ADC) was lowest in the HF12.5 diet and was associated with higher nitrogen fecal losses, but N gain was similar among diets. Phosphorus ADC was significantly improved with the dietary incorporation of HF, decreasing fecal P losses into the environment. Fish fed the diets containing HF grew as well as those fed the FM diet. The feed intake, feed conversion ratio, and protein efficiency ratio were statistically similar among dietary treatments. The protein and lipid whole body composition, as well as the muscle long-chain omega-3 fatty acid profile, remained unaffected by the fishmeal replacement. The immunological parameters assessed (lysozyme, peroxidase and alternative complement pathway) were not affected by the HF inclusion.

The present study shows that an inclusion of up to 12.5% HF (76% fishmeal replacement) is possible in European seabass without impairing feed intake, growth, immune response or EPA and DHA levels in the muscle. Although energy and protein apparent digestibility coefficients (ADC) were lowest in the HF12.5 diet, energy and nutrient gain remained similar among dietary treatments. Altogether results suggest that HF is an interesting ingredient to be used in practical feed formulations for this species.

Introduction

Since July 1st 2013, the use of non-ruminant land animal meals in aquafeeds has been re-allowed in Europe (European Commission, 2013), enabling the incorporation of animal by-products as nutrient sources, which allows the production of economically sustainable aquafeeds and increases the commercial value of these by-products (Klinger and Naylor, 2012; Naylor et al., 2000; 2009). The poultry production industry generates large amounts of by-products, with a yearly production of about 175,000 tons of feather meal in Europe alone (Adler et al., 2014), but this ingredient is currently poorly used as a protein source in aquafeeds. Hydrolyzed feather meal (HF) is a rich protein source – 74 to 91% crude protein (CP; Baker et al., 1981; Bureau et al., 1999; 2000; Grazziotin et al., 2008; Klemesrud et al., 2000), rich in cystine (4 – 5% CP), but deficient in lysine (2%) and methionine (1% CP; Baker et al., 1981; Klemesrud et al., 2000). HF is a good source of proteases, polyunsaturated fatty acids and antioxidant peptides, and has a low content of ash and phosphorus, allowing low water pollution formulations (Lasekan et al., 2013; Sugiura et al., 2000). When obtained from bacterial hydrolysis, HF also shows antihypertensive activities (Fontoura et al., 2014). However, the low lysine and methionine and high cystine content of HF, compared to fishmeal (NRC, 2011), may cause an imbalance and reduce the dietary energy available for fish growth due (Klemesrud et al., 1997; Wang and Parsons, 1998).

The nutrient availability of HF can vary according to the rendering process applied to the feathers. Previous studies reported that protein apparent digestibility coefficient (ADC) of HF is high in Australian silver perch (*Bidyanus bidyanus*, 93%), a freshwater species (Allan et al., 2000), but reduced in salmonids such as Coho salmon (*Oncorhynchus kisutch*, 80%) and rainbow trout (77 – 86%; Bureau et al., 1999; Cheng et al., 2004; Sugiura et al., 1998), and in marine species such as gilthead seabream (*Sparus aurata*, 22%), turbot (*Psetta maxima*, 47%), Atlantic cod (*Gadus morhua*, 62%), European seabass (*Dicentrarchus labrax*, 67%) and rockfish (*Sebastes schlegeli*, 79%; Davies et al., 2009; Lee, 2002; Tibbetts et al., 2006). Enzyme treated feather meal seems to have improved protein and essential amino acid ADC when compared to steam hydrolyzed feather meal in shrimp (Mendoza et al., 2001). In European seabass, crude protein ADC was similar between enzyme treated and steam hydrolyzed feather meal, but the ADC of some amino acids were altered (Davies et al., 2009). Campos et al. (2018) have recently observed that in European seabass the ADC of methionine and serine were improved in the enzyme treated feather meal, but in another study the ADC of lysine and methionine decreased, while arginine and histidine digestibility increased (Davies et al., 2009). The use of HF as fishmeal replacement was shown to be possible up to 25% in malabar grouper (*Epinephelus malabaricus*; Li et al., 2009), 30% in rainbow trout (Bureau et

al., 2000), 40% in Japanese flounder (Kikuchi et al., 1994), 50% in major carp (*Labeo rohita*) fry (Hasan et al., 1997), and 66% in Nile tilapia (*Oreochromis niloticus*) fry (Bishop et al., 1995) without impairing growth. Above these replacement rates for each species, reduced feed intake, weight gain and whole-body protein content were observed, which could be due to the amino acid imbalance and low palatability and digestibility of HF, as reviewed by Yu (2008). A blend of HF with other protein meals was suggested to achieve adequate growth performance and lower feed cost.

Growth performance and nutrient utilization are the criteria most often used to evaluate alternative protein sources in aquafeeds, whereas changes in immune parameters are often overlooked. Zhang et al. (2014) have recently reported less stress in hybrid tilapia (*Oreochromis niloticus x O. aureus*) fed 12% of HF in replacement of soybean or cotton meal. In Atlantic salmon (*Salmo salar*), the replacement of 44% fishmeal by poultry HF had no effects on the immune function (Bransden et al., 2001).

European seabass is one of the most important marine fish species produced in Europe and in the Mediterranean. Its market price depends on production costs, which are deeply associated with the price of aquafeeds, comprising about 50% of the total costs of aquaculture production (Rana et al., 2009). Thus, any fluctuation in the price of the feedstuffs has an important economic impact in the final feed price and in seabass production costs. The inclusion of locally produced HF could significantly reduce the carbon footprint and the environmental impact of aquafeeds (Klinger and Naylor, 2012; Naylor et al., 2009) and may have important repercussions for both the companies that produce and the ones that use such feedstuff. However, there is very little information available on the use of HF in practical diet formulations for European seabass and new studies are hence warranted since these ingredients have recently been allowed in fish feed in EU.

The present study was conducted to evaluate the effect of replacing fishmeal by increasing levels of HF on the nutrient utilization, growth performance and muscle fatty acid composition of European seabass juveniles. The humoral non-specific immune parameters were also determined in plasma after 18 weeks of feeding the experimental diets.

Materials and methods

The present study was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) and conducted according to the European Economic Community animal experimentation guidelines, Directive 2010/63/EU.

Hydrolyzed feather meal and experimental diets

The steam hydrolyzed feather meal (HF) used in this study was obtained from Avicasal - Sociedade Avícola S.A. (Portugal). Poultry feathers were mixed with poultry blood from the slaughtering process (the mix contained about 25% blood) and subjected to steam/pressure hydrolysis (133 ºC; 304 kPa, held for at least 25 min).

Table 13. Chemical composition and amino acid profile of the used hydrolyzed feather meal.

Chemical composition	
Dry matter (DM, %)	94.8
Crude protein (% DM)	84.2
Crude fat (% DM)	10.4
Gross energy (kJ g^{-1} DM)	23.4
Ash (% DM)	3.0
Phosphorus (% DM)	0.4
Amino acids (g 16 g^{-1} N)	
Arginine	7.0
Histidine	1.4
Lysine	3.2
Threonine	4.5
Isoleucine	4.9
Leucine	7.6
Valine	7.4
Methionine	1.5
Phenylalanine	5.6
Cystine	3.0
Tyrosine	3.0
Aspartic acid + Asparagine	6.7
Glutamic acid + Glutamine	10.3
Alanine	6.0
Glycine	7.6
Proline	8.7
Serine	8.4
Taurine	0.9
Hydroxyproline	0.5

Based on the known nutritional requirements of European seabass, four isolipidic (19% DM) and isonitrogenous (50% DM) diets were formulated. A practical feed formulation, based on feedstuffs commonly used by the feed industry, with 32% fishmeal and no HF added, was used as control (FM). Three experimental diets were formulated with increasing levels of HF (5, 7.5 and 15%, HF5, HF7.5 and HF15, respectively), leading to a replacement of about 28,

55 and 76% fishmeal compared to the FM diet. Diets were supplemented with L-Lysine and DL-Methionine to meet the amino acid requirements of this species (Kaushik, 1998). Since HF has a higher protein content and a lower lipid content than the replaced fishmeal (Table 13), the contents of hemoglobin powder, corn meal and fish oil had to be slightly increased with increasing levels of HF to keep diets isoenergetic and isonitrogenous, whilst avoiding dietary supplementation with other amino acids besides lysine and methionine. The ingredient and proximate composition of the experimental diets are shown in Table 14 and the dietary fatty acid and amino acid profiles are in Table 15. Diets were manufactured by SPAROS, Lda. (Portugal), by means of a pilot-scale twin-screw extruder (CLEXTRAL BC45, France) with a screw diameter of 55.5 mm and a temperature range of 105 – 110 °C. Pellet size was 2.0 mm. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 3 h at 60 °C. Following drying, pellets were allowed to cool at room temperature, and subsequently the oil fraction was added under vacuum conditions in a Pegasus vacuum coater (PG-10VCLAB, DINNISEN, The Netherlands).

Table 14. Formulation and proximate composition of the experimental diets.

Ingredients (%)	FM	HF5	HF7.5	HF12.5
Fishmeal Super Prime [1]	2.5	2.5	2.5	2.5
Fishmeal 60 [2]	29.0	20.3	15.0	5.0
Hydrolyzed feather meal [3]	0.0	5.0	7.5	12.5
Hemoglobin powder [4]	2.2	2.5	3.0	4.0
Poultry meal 67 [5]	28.0	28.0	28.0	28.0
Corn gluten [6]	8.2	8.2	8.2	8.2
Sunflower meal [7]	7.5	7.5	7.5	7.5
Corn meal [8]	5.6	8.6	10.6	12.0
Pea starch [9]	3.0	3.0	3.0	3.0
Fish oil [10]	11.7	11.8	11.8	12.1
Vit & Min Premix [11]	1.0	1.0	1.0	1.0
Brewer's yeast [12]	0.4	0.4	0.4	0.4
Binder [13]	0.5	0.5	0.5	0.5
Antioxidant powder [14]	0.2	0.2	0.2	0.2
Sodium propionate [15]	0.1	0.1	0.1	0.1
DCP [16]	0.0	0.0	0.0	2.0
L-Lysine [17]	0.0	0.3	0.5	0.7
DL-Methionine [18]	0.1	0.1	0.2	0.3
Chemical composition				
Dry matter (DM, %)	93.1	93.5	93.0	94.8
Crude protein (% DM)	50.4	49.8	49.9	49.9
Crude fat (% DM)	18.7	19.3	19.3	19.4
Gross Energy (kJ g^{-1} DM)	20.6	21.4	21.6	21.9
Ash (% DM)	16.7	13.6	12.1	10.0
Phosphorus (% DM)	2.0	1.7	1.5	1.5

The abbreviations for the experimental diets stand for: FM – fishmeal-based diet; HF5, HF7.5 and HF12.5 – diets with 5, 7.5 and 12.5 steam hydrolyzed feather meal, respectively;
[1] Peruvian fishmeal LT: 71.0% crude protein (CP), 11.0% crude fat (CF), EXALMAR, Peru;
[2] Fair Average Quality (FAQ) fishmeal: 62.0% CP, 12.0% CF, COFACO;
[3] Hydrolyzed feather meal: 84.2% CP, 10.4% CF, AVICASAL SA;
[4] Hemoglobin powder: 100.0% CP, AP310;
[5] Poultry by-product meal: 69.1% CP, 13.7% CF, SAVINOR SA;
[6] Corn gluten meal: 61.0% CP, 6.0% CF, COPAM, Portugal;
[7] Defatted sunflower meal: 24.0% CP, 1.4% CF, Premix Lda;

[8] Corn meal, Premix Lda, Portugal;
[9] Pea starch, ESASA S.A., Spain;
[10] Fish oil, COPPENS International, The Netherlands;
[11] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings;
[12] Brewer's yeast, Premix Lda, Portugal;
[13] Kielseguhr (natural zeolite): LIGRANA GmbH, Germany;
[14] Antioxidant powder, Paramega PX, Kemin Europe NV, Belgium;
[15] Sodium propionate, Premix Lda, Portugal;
[16] Dicalcium phosphate:18% phosphorus, 18% calcium, Fosfitalia, Italy;
[17] L-Lisine HCl 99%: Ajinomoto Eurolysine SAS, France;
[18] DL-Metionine 99%: Evonik Degussa GmbH, Germany.

Growth trial

The growth trial was conducted in the Fish Culture Experimental Unit of CIIMAR, Porto, Portugal, with juvenile European sea bass (*Dicentrarchus labrax*) obtained from a commercial fish farm (Tinamenor S.L., Cantabria, Spain). In order to adapt to the experimental conditions, fish were kept in quarantine for 4 weeks and fed with a commercial-based diet (AQUASOJA – 49% crude protein, 20% crude fat). After acclimatization, fish were individually weighed (g) and measured (total length, cm) and twelve homogeneous groups of 25 fish (mean body weight 17 g, mean body length 11 cm) were randomly distributed by 55 L tanks within a saltwater recirculation system. Fish were adapted to the new conditions for 2 days (water temperature of 21 ± 1 ºC, salinity of 35 ‰, flow rate at 2 L min^{-1} and 12 h light/12 h dark photoperiod regime) and then each diet was randomly assigned to triplicate fiberglass tanks and fed until apparent satiation, three times a day, by automatic feeders. The amount of feed supplied to each tank was adjusted daily based on the presence or absence of uneaten feed in the tank (Borges et al., 2009; Cabral et al., 2013). Nitrogenous compounds and pH were monitored during the trial and kept at levels recommended for marine species.

The trial lasted 18 weeks, and fish were bulk weighed twice during this period (at 7 and 11 weeks) to monitor and register feed consumption and weight gain. Before the growth trial began, 10 fish from the initial fish stock were collected after a 24 h fasting period, sacrificed by anesthetic overdose (150 mg L^{-1} of MS222; Sigma-Aldrich Co. LLC, Bellfonte, USA), and then kept at -20 ºC, until initial whole-body composition was analyzed. At the end of the 18-week period, fish were anesthetized with 75 mg L^{-1} of MS222 and were individually weighed (g) and measured (total length, cm). Blood was collected from the caudal vein of six fish per tank with heparinized syringes, centrifuged (5000 g for 10 min at 4 °C), and the resulting plasma was stored at - 80 °C. These fish were then killed by a sharp blow to the head prior to sampling the

dorsal muscle, which was immediately frozen with liquid nitrogen and then kept at -80 ºC until determination of total lipid and fatty acid profile. Five fish per tank, sacrificed by anesthetic overdose (150 mg L^{-1}), were collected for whole body composition analysis and frozen at -20 ºC until analysis. The remaining fish were used to evaluate the *in vivo* digestibility of the experimental diets.

Table 15. Amino acid and fatty acid profiles of the experimental diets.

Amino acids (g 16 g^{-1} N)	FM	HF5	HF7.5	HF12.5
Arginine	7.6	7.4	6.9	7.1
Histidine	2.3	2.2	2.0	2.2
Lysine	5.5	5.7	6.2	6.3
Threonine	3.8	3.8	3.7	3.9
Isoleucine	3.6	3.7	3.5	3.6
Leucine	7.7	7.9	7.8	8.4
Valine	5.0	5.4	5.3	5.7
Methionine	2.6	2.4	2.0	2.1
Phenylalanine	4.4	4.4	4.2	4.6
Cystine	0.4	0.5	0.6	0.6
Tyrosine	3.2	3.2	2.9	3.1
Aspartic acid + Asparagine	7.7	7.5	7.8	7.3
Glutamic acid + Glutamine	13.0	12.9	12.8	12.5
Alanine	6.4	6.1	6.0	5.9
Glycine	9.3	8.6	8.1	7.7
Proline	6.3	6.6	6.4	7.0
Serine	4.8	5.1	5.4	6.0
Taurine	0.6	0.5	0.4	0.4
Fatty acids (g 100g^{-1} total fatty acids)				
14:0	4.7	4.7	4.8	4.9
16:0	16.1	16.2	16.4	16.9
18:0	3.3	3.2	3.2	3.4
16:1*n*-7	6.0	6.0	6.0	5.8
18:1*n*-9	15.0	15.0	15.2	15.2
18:1*n*-7	1.9	1.9	1.9	1.9
20:1*n*-9	6.9	7.1	7.0	7.0
22:1*n*-11	9.7	9.9	9.9	10.0
18:2*n*-6	7.7	7.6	7.7	7.6
18:3*n*-3	1.1	1.1	1.1	1.0
18:4*n*-3	2.5	2.6	2.6	2.5
20:4*n*-6	0.6	0.5	0.5	0.5
20:5*n*-3	7.2	7.0	6.9	6.6
22:5*n*-3	0.6	0.6	0.7	0.6
22:6*n*-3	6.5	6.3	6.3	6.2
∑ SFA [1]	25.9	25.9	26.1	27.0
∑ MUFA [2]	40.9	41.2	41.2	41.2
∑ PUFA [3]	28.8	28.5	28.4	27.6
∑ *n*-3 [4]	18.6	18.4	18.3	17.8
∑ *n*-6 [5]	8.8	8.7	8.7	8.5
∑ *n*-3/ ∑ *n*-6	2.1	2.1	2.1	2.1

[1] Includes 12:0, 13:0, 15:0, 17:0, 20:0, 21:0;
[2] Includes 14:1*n*-5, 17:1*n*-7, 14:1*n*-5;
[3] Includes 16:2*n*-4, 16:3*n*-4, 16:4*n*-1, 18:2*n*-4, 18:3*n*-6, 18:4*n*-1, 20:3*n*-6, 20:3*n*-3, 20:4*n*-3, 21:5*n*-3, 22:5*n*-6;
[4] Includes 20:3*n*-3, 20:4*n*-3, 21:5*n*-3;
[5] Includes 18:3*n*-6, 20:3*n*-6, 22:5*n*-6.

Digestibility trial

The apparent digestibility coefficients (ADCs) of the experimental diets were determined by the indirect method, after the incorporation of 1% chromium oxide, as inert marker, to each experimental diet. Each extruded diet was ground and mixed with the marker before being dry pelleted through a 3.2 mm die at 50 ºC using a laboratory pellet press (CPM, C-300 model, S. Francisco, USA). After the growth trial, two homogeneous groups of 21 fish (mean body weight 52 g) previously fed each experimental diet were randomly distributed by 55 L tanks with individual feces sedimentation columns, in a system specially designed for digestibility studies (Guelph system) according to Cho and Slinger (1979). Fish were subjected to the same rearing conditions used during the growth trial.

The experimental diets supplemented with 1% chromium oxide were fed to the fish until apparent satiation, once a day, for 5 days to adapt to each new diet before the feces collection began. About 30 minutes after feeding, each tank was carefully cleaned to assure that there would be no remains of uneaten feed left in the tanks and the sedimentation column. Feces were collected, every morning before feeding, from the sedimentation column, centrifuged to eliminate excess water, and frozen at -20 ºC. Daily collection of the feces was performed for each experimental diet until obtaining an amount considered enough for chemical analysis (8-10 days). At the end of the trial, feces were freeze-dried prior to analysis.

Amino acid analysis

Total amino acids were analyzed in HF and in the experimental diets. Samples were hydrolyzed (6 M HCl at 116 ºC over 24 h in nitrogen-flushed glass vials) before pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Tryptophan was not determined, since it is partially destroyed by acid hydrolysis. The resultant peaks were analyzed with EMPOWER software (Waters, USA).

Proximate analysis of feed, whole body and feces

Fish collected from each tank were ground, pooled and moisture was determined (105 ºC for 24 h). Afterwards, fish were freeze-dried, ground and homogenized before proceeding to the analysis. HF, the experimental diets and the resulting feces were also ground and homogenized prior to analysis (feces were sifted). All chemical analysis followed AOAC

methods and were performed in duplicate. Samples were analyzed for dry matter (105 ºC for 24 h), ash by combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 500 ºC for 5 h), crude protein (N x 6.25) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany), gross energy was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany), and phosphorous content by digestion at 230 ºC in a Kjeldatherm block digestion unit followed by digestion at 60 ºC in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406). Chromic oxide content of diets and feces was determined according to Bolin et al. (1952).

Total lipid and fatty acid analysis

Total lipids in muscle were extracted and quantified gravimetrically by the method described by Folch et al. (1957) using dichloromethane:methanol (2:1) instead of chloroform:methanol (2:1), before fatty acid determination. The fatty acid methyl esters (FAME) contained in muscle lipid extracts and in the experimental diets were transesterified by acidic methylation (Lepage and Roy, 1986), by adding to each extract 1 mL of anhydrous toluene, 0.6 mg of internal standard solution (1 mL of 0.6 mg C19:0/mL hexane; C19:0, Matreya LLC, Pleasant Gap, PA, USA) and 3 mL of freshly prepared 5% (v/v) acetyl chloride in anhydrous methanol. The tubes containing this mixture were incubated at 100 ºC for 60 min, allowed to cool at room temperature, and FAME recovered in 2 mL of hexane. FAME were then analyzed using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Europe GmbH, Germany), equipped with a flame-ionization detector (GC-FID) and a Omegawax 250 capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Supelco, Bellefonte, USA). Helium was used as carrier gas at 35 cm/s, the split ratio was 1:100 and the injected volume 1.0 µL. The thermal gradient was 150 ºC for 7 min, 150 ºC to 170 ºC at 3 ºC/min and held for 25 min, 170 ºC to 220 ºC at 3 ºC/min and held at 220 ºC for 30 min. The injector and detector temperature were maintained at 250 and 260 ºC, respectively. Fatty acids were identified by comparison with known standard mixtures (Supelco 37 Component FAME Mix, Sigma-Aldrich Co. LLC, Bellfonte, USA; PUFA-3 Mixture, Bacterial Acid Methyl Esters CP Mixture, and GLC-110 Mixture, Matreya LLC, Pleasant Gap, USA). Fatty acid methyl ester contents in feed and muscle tissue were expressed on the % of total FAME basis. During the transesterification process, 1 mg of Nonadecylic acid (C:19) was added to each sample as internal standard to enable the quantification of each FAME as mg g^{-1} sample.

Immune parameters analysis

All immune parameters were analyzed in triplicates on a Power-WaveTM microplate spectrophotometer (BioTek Synergy HT, vermont, USA). Lysozyme activity was determined using a turbidimetric assay based on the method described by Ellis (1990) with minor modifications (Costas et al., 2011; Wu et al., 2007) Total peroxidase activity was measured following the procedure described by Quade and Roth (1997) and Costas et al. (2011). The peroxidase activity (units Ml 1 plasma) was determined by defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) was measured using washed rabbit red blood cells (2.8 x 108 cells mL-1; Probiológica, Belas, Portugal) as target cells in the presence of ethylene glycol tetraacetic acid (EGTA; Sigma) and Mg2+ (MgCl2.6H2O; VWR) as described by Sunyer and Tort (1995).

Calculations

The apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Maynard et al. (1979): dry matter ADC (%) = 100*(1-(dietary Cr_2O_3 level/feces Cr_2O_3 level)) and nutrients ADC (%) = 100*(1-(dietary Cr_2O_3 level/feces Cr_2O_3 level)*(feces nutrient or energy level/dietary nutrient or energy)); Average body weight (ABW) = (final body weight + initial body weight)/2; Digestible nitrogen (N), phosphorus (P), lipids (L) or energy (E) intake = (dry feed consumption x N, P, L (%) or E ($kJ.g^{-1}$) in the diet x ADC N, P, L or E)/ABW/days); N, P, L or E gain = (final carcass N, P, L or E content – initial carcass N, P, L or E content)/ABW/days; N, P, L or E retention efficiency = (N, P, L or E gain/Digestible N, P, L or E intake) x 100; Fecal N, P, L or E losses = crude N, P, L or E intake x (1 – (ADC N, P, L or E/100)); Non-fecal N, P or L losses = crude N, P or L intake – N, P or L gain – Fecal N, P or L losses; Non-fecal E losses = non-fecal N losses x 25 kJ N^{-1}; Metabolizable energy (ME) = digestible energy intake – non-fecal E losses; Total heat loss = ME – energy gain; K, Condition factor = (final body weight/(final body length)^3) x 100; Daily growth index = 100 x ((Final body weight)$^{1/3}$ – (Initial body weight)$^{1/3}$)/days; Voluntary feed intake = 100 x crude feed intake/ABW/day; Feed conversion ratio = dry feed intake/weight gain; Protein efficiency ratio = weight gain/crude protein intake; Hepatosomatic index = 100 x liver weight/body weight; Viscerosomatic index = 100 x weight of viscera/body weight.

Statistical analysis

Data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and transformed whenever required (with y = 1/x) before being submitted to a one-way ANOVA with the statistical program IBM SPSS STATISTICS, 22.0 package, IBM corporation, New York, USA (2011). When this test showed significance, individual means were compared using HSD Tukey Test. In all cases significant differences were considered when $p < 0.05$.

Results

The hydrolyzed feather meal used in the present study had high protein content (84%; Table 13). All experimental diets were isolipidic and isonitrogenous, but the inclusion of HF resulted in a reduction of ash and phosphorus contents (Table 14). The fatty acid composition of the experimental diets and their amino acid profiles are reported on Table 15. The HF inclusion led to an increase in the sum of saturated fatty acids (SFA) and to a reduction in the sum of polyunsaturated fatty acids (PUFA), particularly omega-3 PUFA in the diets.

Table 16. Apparent digestibility coefficients (ADC) and nutrient balances of the experimental diets.

ADC (%)	FM	HF5	HF7.5	HF12.5
Dry matter	71.2 ± 1.0	74.5 ± 0.2	74.2 ± 0.7	73.0 ± 1.3
Protein	91.5 ± 0.4^{a}	91.9 ± 0.2^{a}	91.5 ± 0.3^{a}	90.3 ± 0.0^{b}
Energy	85.0 ± 0.7^{a}	85.5 ± 0.2^{a}	84.2 ± 0.6^{ab}	82.2 ± 0.5^{b}
Lipids	95.3 ± 0.1	94.9 ± 1.0	92.9 ± 0.5	93.4 ± 0.6
Phosphorus	31.4 ± 0.4^{c}	39.9 ± 0.2^{b}	44.4 ± 0.5^{a}	43.8 ± 1.7^{a}
Nitrogen (N) balance (mg 100g ABW^{-1} day^{-1})*				
Digestible N intake (DN)	95.0 ± 4.9	100.9 ± 4.6	93.2 ± 5.5	94.1 ±3.3
N gain	22.6 ± 1.9	23.8 ± 1.0	22.2 ± 0.5	22.8 ± 1.1
NRE [1] (% DN)	23.7 ± 0.9	23.6 ± 1.8	23.9 ± 1.9	24.3 ± 1.7
Fecal N losses	8.9 ± 0.5^{b}	8.8 ± 0.4^{b}	8.7 ± 0.5^{b}	10.1 ± 0.4^{a}
Non-fecal N losses	72.4 ± 3.1	77.1 ± 5.1	71.0 ± 5.9	71.2 ± 3.8
Phosphorus (P) balance (mg 100g ABW^{-1} day^{-1})*				
Digestible P intake (DP)	8.3 ± 0.4^{b}	9.5 ± 0.4^{a}	8.6 ± 0.5^{ab}	8.7 ± 0.3^{ab}
P gain	6.1 ± 0.4	6.4 ± 0.6	5.1 ± 0.4	5.6 ± 0.4
PRE [2] (% DP)	73.9 ± 2.0	67.3 ± 8.5	59.2 ± 6.5	64.3 ± 6.3
Fecal P losses	18.0 ± 0.9^{a}	14.3 ± 0.6^{b}	10.8 ± 0.6^{c}	11.1 ± 0.4^{c}
Non-fecal P losses	2.2 ± 0.2	3.1 ± 0.9	3.5 ± 0.7	3.1 ± 0.7
Lipid (L) balance (g kg ABW^{-1} day^{-1})*				
Digestible L intake (DL)	2.3 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
L gain	1.2 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	1.2 ± 0.04
LRE [3] (% DL)	50.3 ± 4.1	49.3 ± 6.3	49.0 ± 2.4	52.5 ± 0.4
Fecal L losses	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
Non-fecal L losses	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Energy (E) balance (J g ABW^{-1} day^{-1})*				
Digestible E intake (DE)	226.1 ± 11.8	252.4 ± 11.4	231.2 ± 13.7	234.8 ± 8.2
E gain	76.0 ± 5.6	81.9 ± 7.1	73.1 ± 2.4	80.1 ± 2.2
ERE [4] (% DE)	33.6 ± 0.7	32.4 ± 2.6	31.7 ± 0.8	34.1 ± 1.1
Fecal E losses	39.8 ± 2.1	42.9 ± 1.9	43.4 ± 2.6	50.9 ± 1.8
Non-fecal E losses	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.2	1.8 ± 0.1
Metabolizable E	224.3 ± 11.7	250.5 ± 11.3	229.4 ± 13.5	233.0 ± 8.1
Total heat loss	148.3 ± 6.1	168.7 ± 10.7	156.3 ± 11.1	152.9 ± 7.2

Values are presented as mean ± standard deviation (n = 3; n = 2 for determination of nutrient ADC). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). Absence of superscript indicates no significant difference between treatments.
* ABW, average body weight; [1] NRE, Nitrogen retention efficiency; [2] PRE, Phosphorus retention efficiency; [3] LRE, Lipid retention efficiency; [4] ERE, Energy retention efficiency.

All the experimental diets were well digested by the fish (dry matter ADC > 70%), as portrayed in Table 16. The protein ADC of the diet with the highest inclusion of HF (HF12.5) was significantly lower than all the other dietary treatments (90 *vs* 92%). Energy digestibility of the diet HF12.5 was the lowest but did not differ significantly from the diet HF7.5. Phosphorus

digestibility increased concomitantly and significantly with the inclusion of HF (40 to 44% versus 31% in FM diet). Even though nitrogen fecal losses were significantly higher in fish fed the HF12.5 diet, N gain was not affected by the experimental diets (Table 16). There were no significant differences among dietary treatments for the metabolic nitrogen losses. Regarding phosphorus balance, fecal P losses were significantly decreased with the dietary incorporation of HF, but P gain remained similar among dietary treatments. The intake of digestible nutrients was also not affected by the HF inclusion, except in the case of phosphorus digestible intake, which was higher for fish fed the HF5 diet than for the ones fed the FM diet. The nitrogen and phosphorus retention efficiency (% digestible intake) is shown in Table 16 and shows no differences among dietary treatments. Energy and lipid intake, gain and retention remained unaffected by the experimental diets (Table 16).

After 18 weeks of feeding, all groups tripled their initial weight (Table 17) and no mortality was registered during the trial. All diets were well accepted by European seabass, resulting in similar voluntary feed intake among dietary treatments. Final body weight (52 g) and the overall growth performance of the fish were similar among the different experimental diets. The HF inclusion did not significantly affect final whole-body composition of the fish (Table 17). The hepatosomatic and viscerosomatic indexes of fish were also similar among experimental conditions (Table 17).

Table 17. Final growth performance, somatic indexes and whole-body composition (% or kJ g^{-1} of wet weight, WW) of European seabass fed the experimental diets for 18 weeks.

	FM	HF5	HF7.5	HF12.5
Growth				
Initial body weight (g)	16.7 ± 2.7	16.8 ± 2.8	16.7 ± 2.8	16.7 ± 2.7
Final body weight (g)	51.5 ± 11.9	53.2 ± 9.5	50.7 ± 8.8	51.6 ± 9.0
Initial body length (cm)	11.5 ± 0.6	11.4 ± 0.6	11.4 ± 0.6	11.4 ± 0.6
Final body length (cm)	16.7 ± 1.2	16.9 ± 0.9	16.7 ± 1.0	16.7 ± 1.4
Final K [1]	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 1.3
DGI [2]	0.9 ± 0.3	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2
VFI [3]	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
FCR [4]	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
PER [5]	1.3 ± 0.04	1.2 ± 0.06	1.3 ± 0.06	1.3 ± 0.09
HSI [6]	1.4 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.4 ± 0.2
VSI [7]	9.1 ± 1.1	9.2 ± 0.9	9.3 ± 1.5	9.6 ± 1.2
Final whole-body composition [8] (% WW)				
Dry matter	35.3 ± 0.7	36.0 ± 1.0	34.8 ± 1.2	35.7 ± 0.9
Crude protein	17.0 ± 0.1	17.3 ± 0.4	16.9 ± 0.6	17.1 ± 0.6
Crude fat	14.1 ± 1.0	14.6 ± 1.0	13.9 ± 0.9	14.8 ± 0.7
Gross energy (kJ g^{-1})	9.1 ± 0.1	9.4 ± 0.3	8.9 ± 0.2	9.4 ± 0.4
Ash	4.3 ± 0.1	4.4 ± 0.1	4.6 ± 0.4	4.2 ± 0.2
Phosphorus	0.8 ± 0.02^{ab}	0.8 ± 0.04^{a}	0.7 ± 0.04^{b}	0.7 ± 0.03^{ab}

Values are presented as mean ± standard deviation (n = 3). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). Absence of superscript indicates no significant difference between treatments.

[1] K, Condition factor; [2] DGI, Daily growth index; [3] VFI, Voluntary feed intake; [4] FCR, Feed conversion ratio; [5] PER, Protein efficiency ratio; [6] HSI, Hepatosomatic index; [7] VSI, Viscerosomatic index; [8] Initial body composition: dry matter, 34.5%; protein, 16.5%; lipids, 14.1%; energy, 8.7 kJ g^{-1}; ash, 4.4%, phosphorus, 2.2%.

The total lipid content and the fatty acid composition in the muscle were generally similar among fish fed the different experimental diets (Table 18), with only slight but significant differences in the amounts of palmitic acid (16:0), arachidonic acid (20:4*n*-6), cetoleic acid (22:1*n*-11) and stearidonic acid (18:4*n*-3). Furthermore, the muscle levels of EPA (20:5n-3) and DHA (22:6n-3) were similar in all diets.

Table 18. Muscle total lipid content (% wet weight, WW) and fatty acid composition (g 100g^{-1} total fatty acids) of the European seabass fed the experimental diets.

	FM	HF5	HF7.5	HF12.5
Total lipids	3.4 ± 0.6	3.5 ± 0.4	3.4 ± 0.9	3.4 ± 0.5
Fatty acids				
14:0	3.0 ± 0.2	3.1 ± 0.1	3.2 ± 0.2	3.2 ± 0.1
16:0	18.4 ± 0.2^{a}	17.8 ± 0.6^{b}	17.6 ± 0.1^{b}	17.8 ± 0.4^{b}
18:0	3.7 ± 0.3	3.5 ± 0.2	3.5 ± 0.1	3.4 ± 0.1
16:1*n*-7	4.9 ± 0.4	5.0 ± 0.1	5.1 ± 0.2	5.1 ± 0.1
18:1*n*-9	18.6 ± 0.8	19.1 ± 0.6	19.3 ± 0.7	19.3 ± 1.0
18:1*n*-7	2.3 ± 0.10^{ab}	2.3 ± 0.03^{a}	2.3 ± 0.03^{a}	2.2 ± 0.05^{b}
20:1*n*-9	4.5 ± 0.2	4.6 ± 0.2	4.7 ± 0.3	4.7 ± 0.1
22:1*n*-11	4.2 ± 0.1^{b}	4.3 ± 0.3^{ab}	4.5 ± 0.4^{ab}	4.7 ± 0.1^{a}
18:2*n*-6	6.5 ± 0.3	6.7 ± 0.2	6.8 ± 0.2	6.8 ± 0.1
18:3*n*-3	0.8 ± 0.07	0.9 ± 0.02	0.9 ± 0.02	0.8 ± 0.02
18:4*n*-3	1.3 ± 0.04^{b}	1.4 ± 0.10^{a}	1.4 ± 0.08^{a}	1.5 ± 0.03^{a}
20:4*n*-6	1.0 ± 0.1^{a}	0.9 ± 0.1^{ab}	0.9 ± 0.1^{ab}	0.8 ± 0.1^{b}
20:5*n*-3	7.3 ± 0.1	7.1 ± 0.1	7.3 ± 0.3	7.0 ± 0.1
22:5*n*-3	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
22:6*n*-3	12.0 ± 1.0	11.7 ± 0.4	11.8 ± 1.1	11.1 ± 0.6
EPA + DHA [1]	7.3 ± 0.3	7.0 ± 1.1	6.5 ± 0.5	6.9 ± 0.3
∑ SFA [2]	26.8 ± 0.4^{a}	26.0 ± 0.6^{b}	25.7 ± 0.3^{b}	26.0 ± 0.5^{b}
∑ MUFA [3]	35.5 ± 1.2	36.4 ± 0.9	36.9 ± 1.3	37.0 ± 1.2
∑ PUFA [4]	32.6 ± 0.7	32.4 ± 0.3	32.5 ± 1.2	31.6 ± 0.8
∑ *n*-3 [5]	23.4 ± 1.0	23.2 ± 0.4	23.1 ± 1.3	22.4 ± 0.9
∑ *n*-6 [6]	8.4 ± 0.2	8.4 ± 0.2	8.5 ± 0.1	8.4 ± 0.1
∑ *n*-3/ ∑ *n*-6	2.8 ± 0.2	2.8 ± 0.1	2.7 ± 0.2	2.7 ± 0.1

[1] EPA + DHA = eicosapentaenoic acid + docosahexaenoic acid, expressed in mg g^{-1} muscle wet weight; [2] Includes 12:0, 13:0, 15:0, 17:0, 20:0, 21:0; [3] Includes 14:1n-5, 17:1n-7, 14:1n-5; [4] Includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-6, 18:4n-1, 20:3n-6, 20:3n-3, 20:4n-3, 21:5n-3, 22:5n-6; [5] Includes 20:3n-3, 20:4n-3, 21:5n-3; [6] Includes 18:3n-6, 20:3n-6, 22:5n-6.Values are presented as mean ± standard deviation (n = 3). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). Absence of superscript indicates no significant difference between treatments.

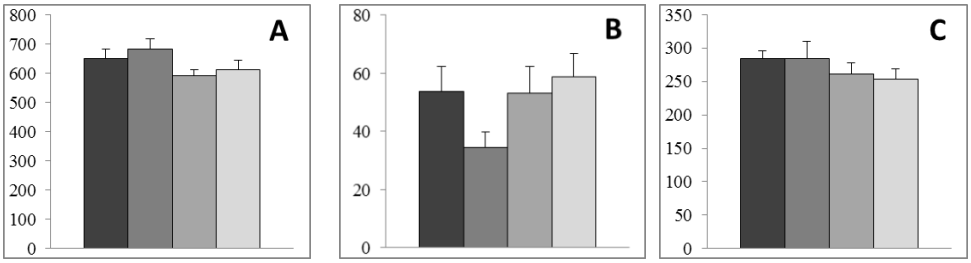

Figure 11. Effects of the experimental diets (■ - FM, ■ - HF5, ■ - HF7.5, □ - HF12.5) on humoral non-specific parameters of European seabass after 18 weeks. A: lysozyme (EU min^{-1} mL^{-1}); B: peroxidase (EU mL^{-1}); C: ACH50 (units mL^{-1}). Values are presented as mean ± standard error (n = 18). Absence of superscript letter indicates no significant difference between treatments ($p > 0.05$).

The humoral non-specific immune parameters evaluated in plasma (peroxidase, lysozyme and alternative complement pathway) were not significantly affected by the HF inclusion (Figure 11).

Discussion

Every diet used in this trial was very well digested by the European seabass. The protein apparent digestibility coefficient (ADC) of the diet with highest fishmeal replacement (HP12.5) was significantly decreased (90% *vs* 92% in the FM diet), which led to significantly higher nitrogen fecal losses in fish fed that diet (10 mg 100g ABW^{-1} day^{-1}). A previous study showed that, in gilthead seabream, a replacement of 19 and 25% fishmeal by hydrolyzed feather meal (HF) resulted in a reduction of protein ADC from 75 to 67%, respectively, compared to 92% in the control diet (Nengas et al., 1999). Phosphorus ADC was significantly improved in the present study, contrarily to N, with the replacement of fishmeal by HF (40 – 44% in HF diets *versus* 31% in FM). This higher digestibility allied with the lower P content of the HF, resulted in significantly lower fecal P losses in fish fed the diets with HF (11 – 14 mg 100g ABW^{-1} day^{-1}). These results suggest that diets with the inclusion of HF could help prevent effluents pollution by releasing low amounts of P into the environment, as previously suggested by Sugiura et al. (2000) for rainbow trout. However, high inclusions of HF (> 7.5%) may also result in a higher N discharge into the water, that if not properly treated could result in the pollution of aquaculture effluents. The energy and lipid balance were not affected by the HF inclusion, being within the range of values previously reported for this species with fishmeal-based diets (Peres and Oliva-Teles, 1999; 2005; Santos, 2010; Valente et al., 2006), suggesting that the fishmeal replacement did not impair the energy or the lipid utilization.

The replacement of fishmeal in European seabass diets has been successfully accomplished by using vegetable sources (up to 98%; Kaushik et al., 2004) or single cell proteins (up to 50%; Oliva-Teles and Goncalves, 2001). However, to our best knowledge, no literature is available on the effects of using HF as fishmeal replacement on the growth performance of this fish species. Nonetheless, the use of this ingredient as fishmeal replacement in other fish species has been previously shown by several authors to be limited to very low levels of inclusion, mainly due to its unbalanced amino acid profile and low palatability, which causes decreased feed intake and reduced weight gain (reviewed by Yu (2008)). In the present study, and contrarily to previous reports (Fasakin et al., 2005), voluntary feed intake was similar for every diet, indicating that HF meal induced a food palatability that did not interfere with fish feed consumption. The HF used was a blend of poultry feathers and poultry blood, which might have enhanced HF acceptability by the fish, surpassing the palatability issues of this ingredient, as well as other nutritional setbacks.

The majority of previous studies evaluating the feasibility of HF in fish did not balance the amino acid profile of the diets, which could mainly explain growth impairment. In the present study, crystalline amino acids were supplemented to the experimental diets in order to achieve

the recommended values for European seabass. At the end of the growth trial, no differences were observed among dietary treatments on growth performance or whole-body composition even at 76% fishmeal replacement (HF12.5). This inclusion level is highly above previously reported successful values of HF as fishmeal replacement. Previous studies have shown that the replacement of fishmeal with HF above 25% in Malabar grouper, 30% in rainbow trout, 40% in Japanese flounder, 50% in major carp and 66% in Nile tilapia led to reduced growth performances and decreased whole body protein content (Bishop et al., 1995; Bureau et al., 2000; Hasan et al., 1997; Kikuchi et al., 1994; Li et al., 2009). Moreover, dietary supplementation with crystalline amino acids showed only limited improvement on the growth performance of Japanese flounder (Kikuchi et al., 1994) and rainbow trout (Pfeffer et al., 1994). In rainbow trout, there was a reduction of the feed intake concomitant with the fishmeal replacement, which was probably responsible for the growth reduction, but in Japanese flounder, even though the amino acid requirements were fulfilled, there was an increase in feed intake and a reduction in growth associated with the HF inclusion. The good growth performances obtained in the present study are probably a result of both the dietary supplementation of crystalline amino acids, ensuring the required essential amino acids for this species were fulfilled, and the good palatability of the experimental diets.

The total lipid content in the dorsal muscle of fish fed the experimental diets ranged from 3.4 to 3.5% wet weight and was similar among dietary treatments ($p > 0.05$). Furthermore, the muscle fatty acid profile of European seabass generally reflected the dietary fatty acid profile and seems to be little affected by the fishmeal supplementation. Muscle of the fish fed HF12.5 had significantly lower amounts of arachidonic acid (20:4*n*-6) and significantly higher amounts of cetoleic acid (22:1*n*-11) than fish fed FM, clearly reflecting the dietary levels of these fatty acids. But the amount of stearidonic acid (18:4*n*-3) was significantly higher in the muscle of fish fed diets containing HF, even though this fatty acid was present in similar amounts in all diets. On the other hand, significant decreases in palmitic acid (16:0) and in the sum of saturated fatty acids were obtained for fish fed HF diets, when compared to fish fed FM, even though those diets contained higher amounts of these fatty acids compared to the FM diet. These results suggest that these fatty acids were either preferentially hydrolyzed by these fish or that there is a preferential deposition in other organs than the dorsal muscle, as previously suggested by other authors (Jezierska et al., 1982; Raclot and Groscolas, 1994). Moreover, the amounts of eicosapentaenoic and docosahexaenoic acid in the muscle were statistically similar among dietary treatments, despite these fatty acids being present in lower amounts in the diets with HF inclusion, suggesting a preferential deposition of these fatty acids into this tissue. All fish had high levels of omega-3 fatty acids in the muscle (6.5 to 7.3 mg g^{-1}), which are well above the range of the recommended levels for human consumption to

decrease the risk of cardiovascular diseases (0.25 – 0.5 g per 100 g portion of fish; Authority, 2010), demonstrating its great nutritional value for human consumption.

The humoral non-specific immune parameters of European seabass analyzed in this study – lysozyme, peroxidase and ACH50 activity, common indicators of the innate immune status in fish, were not significantly affected by the HF inclusion in the diets. Even though vegetable protein sources have been previously studied and reported to alter fish immune response (Krogdahl et al., 2000; Rumsey et al., 1994; 1995), the impact of land animal protein sources in aquafeeds has been poorly evaluated (Neji et al., 1993). The present study shows that the replacement of fishmeal by HF up to 76% has no effects on the immune function, supporting previous results in Atlantic salmon after a 44% fishmeal substitution (Bransden et al., 2001).

The results obtained in this study showed that an inclusion of HF up to 12.5% in European seabass diets (replacing 76% of the fishmeal in the diet) is possible without impairing either growth, immune status or EPA and DHA levels in the muscle, suggesting that this can be a very good protein source to incorporate in feeds for this species. Phosphorus ADC was significantly improved with the dietary incorporation of HF, decreasing fecal P losses into the environment. Although energy and protein apparent digestibility coefficients (ADC) were lowest in the HF12.5 diet, energy and nutrient gain remained similar among dietary treatments. Furthermore, since this ingredient was obtained by rendering locally produced by-products, its use could help reduce the need for imported feedstuffs, thus diminishing the carbon footprint of the aquafeed sector.

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Chapter IV

Ability of European seabass (*Dicentrarchus labrax*) to digest rendered animal fats from fish, poultry and mammals

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Ability of European seabass (*Dicentrarchus labrax*) to digest rendered animal fats from fish, poultry and mammals

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Ability of European seabass (*Dicentrarchus labrax*) to digest rendered animal fats from fish, poultry and mammals

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Abstract

This study evaluates the *in vivo* apparent digestibility coefficients (ADC) of rendered animal by-product fats in European seabass (*Dicentrarchus labrax*) juveniles. Diets contained fish oil (FOd), poultry fat (PFd) or mammal fat (MFd), from rendering animal by-products, at 140 g/kg. Macronutrient and fatty acids ADCs were evaluated in quadruplicate tanks with fifteen 100 g fish. While total lipids ADCs (88 – 90%) were unaffected, individual fatty acids ADC varied significantly among diets. Monounsaturated fatty acids ADCs were similar (94 – 95%), saturated fatty acids ADCs were lowest in MFd (85 *vs* 91% in PFd and FOd) and polyunsaturated fatty acids (PUFA) ADCs were lowest in PFd (96 *vs* 97 – 98%). Total *n*-3 PUFA were better digested in FOd than in the other diets (98 *vs* 96 – 97%), while MFd had the highest total *n*-6 PUFA ADC. FOd showed significantly higher EPA (20:5*n*-3) and DHA (22:6*n*-3) ADCs compared to the other test diets. The obtained results suggest that rendered animal fats can be considered feasible alternatives to fish oil in seabass. Nevertheless, that lack of EPA and DHA in land animal fats, combined with the reduced digestibility of these fatty acids, may compromise its dietary inclusion at high levels.

Introduction

The production of European seabass (*Dicentrarchus labrax*), a marine fish species with a high economic impact in Mediterranean countries, highly depends on aquafeed prices, which comprise over 50% of finfish total production costs (Rana et al., 2009). Marine fish oils have been the main dietary source of essential fatty acids for carnivorous fish, but the depletion of wild stocks and increasing fish oil prices have made this ingredient economically and environmentally unsustainable (Klinger & Naylor, 2012). Fish oil is a major source of long-chain highly unsaturated fatty acids (LC-HUFA), such as 20:5*n*-3 (EPA) and 22:6*n*-3 (DHA), which are important to promote optimal growth and health of farmed marine fish (Sargent et al., 2002). These fatty acids are also associated with the prevention of cardiovascular and inflammatory diseases in humans (Ulbricht & Southgate, 1991). Replacing fish oil in aquafeeds is not an easy task as most available alternatives only have, at most, vestigial levels of EPA and DHA (Trushenski & Lochmann, 2009). Rendered fats, obtained by processing animal by-products, have a relatively low price, are widely available in the market and can be considered valid alternatives to fish oil. Large amounts of rendered fats are produced annually in Europe (12 Mt; EPFRA (2016)) and their valorization could bring economic benefits for both the companies that produce them and the aquafeed producers. Furthermore, the use of rendered fats could significantly reduce the environmental impact associated with aquafeeds through the decrease of net primary production use (Papatryphon et al., 2004). Also, according to the Scientific Steering Committee, there is no evidence that tallow derived from ruminant animals would constitute a TSE (transmissible spongiform encephalopathy) risk, since TSE agents are associated with protein impurities that may be present in the end-product. Therefore, tallow should be safely used in feed production (except from calf feed) and pet food, if it is purified to levels below 1.5 g/kg insoluble impurities (SSC, 2001).

Fish oil is extremely rich in polyunsaturated fatty acids (PUFA), being more susceptible to oxidation than land animal fats such as pork lard, beef tallow and poultry fat, which are richer in saturated (SFA) and monounsaturated fatty acids (MUFA; Watanabe (1989)). When obtained from fish by-products (from canning industries), fish oil is still an excellent source of *n*-3 HUFA, being more environmentally sustainable than fisheries-derived fish oil (Laso et al., 2016). Compared to fish oil, poultry fat has a higher alpha-linolenic acid (18:3*n*-3) content and more *n*-6 PUFA, particularly linoleic acid (18:2*n*-6), even though it has a lower amount of *n*-3 PUFA (Emery et al., 2014). Beef tallow, on the other hand, has very high amounts of SFA, particularly 16:0 and 18:0, and both land animal fats have higher MUFA content than fish oil, particularly 18:1*n*-9 (Emery et al., 2014). The energy and fatty acids availability of these land animal fats highly depends on their digestibility, which has been shown to decrease in SFA-

rich ingredients (Hua & Bureau, 2009). SFA and MUFA have higher melting points than PUFA and therefore poultry and mammal fats are usually solid at room temperature (melting points above 23 and 34 ºC, respectively; Alm (2013)), while fish and vegetable oils, richer in PUFA, are usually liquid under these conditions. Since chemical and enzymatic digestive processes are highly related to surface area, solidification may have dramatic effects on the digestibility and absorption of rendered fats (Trushenski & Lochmann, 2009). Furthermore, previous studies have suggested a preferential hydrolysis of triacylglycerol ester bonds with PUFA in fish gut, compared to SFA and MUFA, and an increase in enterocyte uptake related to the chain length and degree of unsaturation of fatty acids (Oxley et al., 2005). However, Sibbald et al. (1961) have reported that PUFA have a synergistic effect on SFA digestibility, indicating that some dietary PUFA can enhance the digestibility of SFA rich fats.

There is very little information available regarding the digestibility of rendered fats by fish, and its use in European seabass diets has never been assessed, since more attention has been given towards using vegetable oils as fish oil substitutes for this species (Izquierdo et al., 2003). A broader assessment of the digestibility of different dietary fatty acid sources by fish is required to evaluate the potential of using alternative fat sources as fish oil replacers. Several authors have already reported poultry fat and beef tallow as feasible alternative lipid sources for Atlantic salmon (*Salmo salar*), resulting in diets with high palatability and that produce high growth performance and a good final product (Emery et al., 2014). Nevertheless, in Atlantic halibut (*Hippoglossus hippoglosssus*) at 12 ºC, poultry fat has been reported to have a reduced MUFA and PUFA digestibility compared to fish oil, even though the total lipid ADC was similar in both fat sources (Martins et al., 2009). On the other hand, Emery et al. (2014) reported decreased lipid digestibility in Atlantic salmon when more than 300 g/kg poultry fat was replaced by beef tallow, even though growth performance was not affected.

In the present study, three rendered animal fats were selected to assess the *in vivo* apparent digestibility coefficients of macronutrients and fatty acids in European seabass juveniles. Fish oil (FO) from rendering fish by-products (skin, meat and viscera), poultry fat (PF) and mammal fat (MF, a mixture of about 700 g/kg lard and 300 g/kg beef tallow) from rendering either poultry or mammal by-products (viscera, skin, bruised meat and bones) were added at 140 g/kg to a practical feed formulation blend commonly used by the feed industry.

Materials and methods

The present study was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

Ingredient origin and experimental diets

Table 19. Chemical composition and fatty acid profile of the supplemental lipid sources used (FO - fish oil; PF - poultry fat; MF - mammal fat).

Chemical composition	FO	PF	MF
Dry matter (DM, %)	99.73	100.00	100.00
Total lipid (% DM)	88.45	86.79	88.85
Gross energy (kJ g^{-1} DM)	39.63	39.98	40.00
Acidity (%)	7.9	4.82	4.18
Peroxide value (mEq $O_2.kg^{-1}$)	5.7	ND[6]	ND[6]
Insoluble impurities (%)	0.18	0.70	0.02
Fatty acids (% total fatty acids)			
14:0	3.91	0.52	1.62
16:0	17.70	21.27	23.75
18:0	4.88	5.67	14.02
16:1*n*-7	5.37	4.92	2.52
18:1*n*-9	14.84	36.68	37.52
18:1*n*-7	2.56	1.84	3.55
20:1*n*-9	1.93	0.24	0.68
18:2*n*-6	2.01	23.79	10.60
18:3*n*-3	0.57	1.50	0.55
20:5*n*-3	8.51	0.04	0.03
22:5*n*-3	1.65	0.04	0.08
22:6*n*-3	19.06	0.00	0.00
∑ SFA [1]	29.96	27.91	41.14
∑ MUFA [2]	27.63	44.00	44.85
∑ PUFA [3]	37.60	26.99	12.78
∑ *n*-3 [4]	32.03	1.70	0.92
∑ *n*-6 [5]	4.54	25.20	11.75
∑ *n*-3/ ∑ *n*-6	7.06	0.07	0.08

[1] ∑ SFA is the sum of saturated fatty acids, which also includes 12:0, 13:0, 15:0, 17:0, 20:0, 21:0;
[2] ∑ MUFA is the sum of monounsaturated fatty acids, which also includes 14:1n-5, 17:1n-7, 22:1n-11;
[3] ∑ PUFA is the sum of polyunsaturated fatty acids, which also includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-6, 18:4n-1, 18:4n-3, 20:3n-6, 20:3n-3, 20:4n-3, 20:4n-6, 21:5n-3, 22:5n-6;
[4] ∑ n-3 is the sum of n-3 PUFA;
[5] ∑ n-6 is the sum of n-6 PUFA;
[6] ND, Non detected (bellow detection level).

The fish oil (FO), poultry fat (PF) and mammal fat (MF) used in this study were obtained from a Portuguese company specialized in the collection, treatment and valorization of animal by-products – SAVINOR S.A. (Portugal). FO was obtained by rendering fish by-products from fisheries and mainly from the tuna canning industry (skin, meat and viscera). PF and MF were obtained by rendering either poultry or mammal (a mixture of 700 g/kg pork and 300 g/kg beef) by-products from local slaughterhouses (viscera, skin, bruised meat and bones). The chemical composition and fatty acid profile of each fat source is presented in Table 19. To create a practical feed formulation blend commonly used by the feed industry, FO, PF and MF were included to the experimental diets at 140 g/kg (FOd, PFd and MFd; Table 20). All diets were isoenergetic (23 MJ/kg) and isonitrogenous (480 g/kg crude protein) and had 5-α cholestane (0.7 g/kg) included as inert marker for determination of fatty acids digestibility, as previously suggested by Sigurgisladottir et al. (1992). Dietary fatty acid profile of the diets is depicted in Table 21. Diets were manufactured by SPAROS, Lda. (Portugal), by means of a pilot-scale twin-screw extruder (CLEXTRAL BC45, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110 °C. Pellet size was 2.0 mm. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 3 h at 60 °C. Following drying, pellets were allowed to cool at room temperature, and subsequently the respective oil fraction was added under vacuum conditions in a Pegasus vacuum coater (PG-10VCLAB, DINNISEN, The Netherlands).

Digestibility trial

The digestibility trial was conducted in the Fish Culture Experimental Unit of CIIMAR (Porto, Portugal), with juvenile European seabass obtained from a commercial fish farm (Tinamenor SL, Cantabria, Spain). Each diet was tested in quadruplicate using new groups of fish. In order to adapt to the experimental conditions (water temperature of 21 ± 1 ºC, salinity of 35 ‰, flow rate at 2 L/min and 12 h light/12 h dark photoperiod regime), twelve homogeneous groups of 15 fish (mean body weight 100 g) were kept in 55 L fiber glass tanks for 4 weeks and fed once a day with a commercial diet (AQUASOJA – 490 g/kg crude protein, 200 g/kg crude fat). Since the system only comprised six tanks with individual feces sedimentation columns (Guelph system), specially designed according to Cho and Slinger (1979), two successive experiments were conducted under the same conditions using different fish to get four independent replicates per diet (N = 4). After acclimatization, each experimental diet was randomly assigned to two of the six tanks.

Fish were fed for 5 days, once a day, until apparent satiation, before starting feces collection, to allow total evacuation of previous diets from the fish digestive system (Alexis et al., 2016). About 30 minutes after feeding, every tank was carefully cleaned to ensure that there would be no remains of uneaten feed left in the tanks or in the sedimentation column.

Feces were collected from the sedimentation column every morning, before feeding, and then centrifuged to eliminate water excess before freezing at -20 ºC. Daily collection of the feces was performed for each experimental diet for 8 to 10 days, following previous seabass digestibility studies (Campos et al., 2017), until obtaining an amount considered enough for chemical analysis. At the end of the trial, feces were freeze-dried prior to analysis.

Table 20. Formulation and proximate composition of the experimental diets (FOd – reference diet, PFd – poultry fat diet, MFd – mammal fat diet).

Ingredients (g/kg)	FOd	PFd	MFd
Fishmeal LT Diamante [1]	25.0	25.0	25.0
Fishmeal 60 [2]	282.0	282.0	282.0
Hemoglobin powder [3]	20.0	20.0	20.0
Poultry meal 65 [4]	100.0	100.0	100.0
Soy protein concentrate [5]	130.0	130.0	130.0
Wheat gluten [6]	40.0	40.0	40.0
Corn gluten [7]	60.0	60.0	60.0
Sunflower meal [8]	60.0	60.0	60.0
Corn meal [9]	90.0	90.0	90.0
Pea starch [10]	30.3	30.3	30.3
Fish oil [11]	138.0	0.0	0.0
Poultry fat [11]	0.0	138.0	0.0
Mammal fat [11]	0.0	0.0	138.0
Vitamin & Mineral Premix [12]	10.0	10.0	10.0
Brewer's yeast [13]	3.2	3.2	3.2
Binder [14]	4.0	4.0	4.0
L-Lysine [15]	6.6	6.6	6.6
DL-Methionine [16]	1.2	1.2	1.2
5α-cholestane	0.7	0.7	0.7
Chemical composition			
Dry matter (DM, g/kg)	949.6	948.6	946.3
Crude protein (g/kg DM)	489.8	481.1	481.9
Crude fat (g/kg DM)	189.5	192.1	199.2
Gross Energy (MJ/kg DM)	22.62	22.91	22.91
Ash (g/kg DM)	122.4	119.7	119.3

[1] Peruvian fishmeal LT: 710 g/kg crude protein (CP), 110 g/kg crude fat (CF), EXALMAR, Peru;
[2] Fair Average Quality (FAQ) fishmeal: 620 g/kg CP, 120 g/kg CF, COFACO, Portugal;
[3] Hemoglobin powder: 1000 g/kg CP, 9 g/kg CF, AP310;
[4] Poultry by-product meal: 691 g/kg CP, 137 g/kg CF, SAVINOR S.A.;
[5] Soy protein concentrate: 650 g/kg CP, 7 g/kg CF, ADM, The Netherlands;
[6] Wheat gluten: 840 g/kg CP, 13 g/kg CF, ROQUETTE, France;
[7] Corn gluten meal: 610 g/kg CP, 60 g/kg CF, COPAM, Portugal;
[8] Defatted sunflower meal: 240 g/kg CP, 14 g/kg CF, Premix Lda, Portugal;
[9] Corn meal, Premix Lda, Portugal;
[10] ESASA S.A., Spain;
[11] Fish oil, poultry fat and mammal fat, SAVINOR S.A., Portugal;
[12] Premix for marine fish, PREMIX Lda., Portugal. Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings;
[13] Brewer's yeast, Premix Lda, Portugal;
[14] Kielseguhr (natural zeolite): LIGRANA GmbH, Germany;
[15] L-Lisine HCl 99%: Ajinomoto Eurolysine SAS, France;
[16] DL-Metionine 99%: Evonik Degussa GmbH, Germany.

Proximate analysis of ingredients, diets and feces

Experimental diets were ground and collected feces sifted and homogenized before proceeding with the analysis. The tested land animal fats were liquefied (at about 35 ºC) and homogenized before being analyzed. Proximate composition analysis was performed according to the Association of Official Analytical Chemists (AOAC, 2006) methods and performed in duplicate. Acidity, peroxide value and insoluble impurities were determined in the tested fats according to EN ISO 660:2002, ISO 3960:2007 and EN ISO 663:2005, respectively. The tested fats, diets and feces were analyzed for dry matter (105 ºC for 24 h) and gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). In the experimental diets and collected feces, crude protein was determined (nitrogen x 6.25) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA), and ash content after combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 500 ºC for 5 h). Dietary crude fat content was quantified after petroleum ether extraction using Soxtec 2055 (Foss, Denmark), to enable direct comparison with other studies. So, before fatty acid determination in lipid sources, diets and feces, total lipids were extracted by the method described by Folch, Lees, and Sloane Stanley (1957) using dichloromethane:methanol (2:1) instead of chloroform:methanol (2:1). The extracts were stored for subsequent fatty acid and 5α-cholestane determination.

Table 21. Fatty acid profile of the experimental diets (FOd - fish oil diet; PFd - poultry fat diet; MFd - mammal fat diet).

Fatty acids (% total fatty acids DM diet)	FOd	PFd	MFd
14:0	3.73	1.15	1.57
16:0	18.07	20.79	21.81
18:0	4.98	5.48	8.84
16:1*n*-7	4.99	3.80	3.80
18:1*n*-9	16.83	34.07	33.83
18:1*n*-7	2.52	2.67	2.59
20:1*n*-9	2.24	1.08	1.09
18:2*n*-6	5.55	21.98	16.80
18:3*n*-3	0.79	1.06	1.07
20:5*n*-3	6.89	0.60	0.63
22:5*n*-3	1.41	0.23	0.22
22:6*n*-3	15.08	0.94	0.99
∑ SFA [1]	29.89	33.21	33.59
∑ MUFA [2]	30.23	43.30	43.00
∑ PUFA [3]	35.74	21.81	21.69
∑ *n*-3 [4]	26.20	3.24	3.40
∑ *n*-6 [5]	8.25	23.44	18.04
∑ *n*-3/ ∑ *n*-6	3.17	0.14	0.19

[1] ∑ SFA is the sum of saturated fatty acids, which also includes 12:0, 13:0, 15:0, 17:0, 20:0, 21:0;
[2] ∑ MUFA is the sum of monounsaturated fatty acids, which also includes 14:1n-5, 17:1n-7, 22:1n-11;
[3] ∑ PUFA is the sum of polyunsaturated fatty acids, which also includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-6, 18:4n-1, 18:4n-3, 20:3n-6, 20:3n-3, 20:4n-3, 20:4n-6, 21:5n-3, 22:5n-6;
[4] ∑ n-3 is the sum of n-3 PUFA;
[5] ∑ n-6 is the sum of n-6 PUFA.

Fatty acid and 5α-cholestane determination

The fatty acid methyl esters (FAME) contained in the lipid extracts obtained were transesterified by acidic methylation (Lepage & Roy, 1986), by adding to each extract 1 mL of anhydrous toluene, 0.6 mg of internal standard solution (1 mL of 0.6 mg C19:0/mL hexane; C19:0, Matreya LLC, Pleasant Gap, PA, USA) and 3 mL of freshly prepared 5% (v/v) acetyl chloride in anhydrous methanol. The tubes containing this mixture were incubated at 100 ºC for 60 min, allowed to cool at room temperature, and FAME were recovered in 2 mL of hexane. FAME were then analyzed using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Europe GmbH, Germany), equipped with a flame-ionization detector (GC-FID) and a Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Supelco, Bellefonte, USA). Helium was used as carrier gas at 35 cm/s, the split ratio was 1:100 and the injected volume 1.0 µL. The thermal gradient was 150 ºC for 7 min, 150 ºC to 170 ºC at 3 ºC/min and held for 25 min, 170 ºC to 220 ºC at 3 ºC/min and held at 220 ºC for 30 min. The injector and detector temperature were maintained at 250 and 260 ºC, respectively. Fatty acids were identified by comparison with known standard mixtures (Supelco 37 Component FAME Mix, Sigma-Aldrich Co. LLC, Bellfonte, USA; PUFA-3 Mixture, Bacterial Acid Methyl Esters CP Mixture, and GLC-110 Mixture, Matreya LLC, Pleasant Gap, USA). 5α-cholestane, used in this trial as inert digestibility marker, was also identified in this process by comparison with a prepared sample of this compound, which was transesterified as described above. Due to the addition of the mentioned internal standard, the amount of 5α-cholestane was also expressed in mg/g of each sample, which allowed the use of this marker in the calculation of the apparent digestibility coefficients of dry matter, protein and energy.

Calculations

The tested diets had 5α-cholestane as inner marker for macronutrient and fatty acids apparent digestibility coefficients (ADCs) determination. ADCs of the experimental diets were calculated according to Maynard, Loosli, Hintz, and Warner (1979) for dry matter: ADC (%) = 100 × (1 − (dietary 5α-cholestane level / feces 5α-cholestane level)); and for macro nutrients and fatty acids: ADC (%) = 100 × (1 − (dietary 5α-cholestane level / feces 5α-cholestane level) × (feces nutrient or energy level / dietary nutrient or energy level)).

Statistical analysis

ADCs data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and transformed with $y = \arcsin(\sqrt{\chi})$ whenever necessary. Data were then submitted to a one-way ANOVA with the statistical program IBM SPSS STATISTICS, 22.0 package, IBM Corporation, New York, USA (2011). When this test showed significance, individual means were compared using HSD Tukey Test. In all cases, significant differences were considered when $p < 0.05$.

Results

The chemical composition and fatty acid profile of each animal fat used in this study is presented in Table 19. All fats had similar total lipid (869 – 888 g/kg DM) and gross energy content (40 MJ/kg DM). The apparent digestibility coefficients (ADCs) of the experimental diets are reported in Table 22. Total lipids ADC (88 – 90%) were similar ($p > 0.05$) among experimental diets. Protein ADC (92 – 93%) was similar between FO diet (FOd) and the other diets, but higher in MF diet (MFd) than in PF diet (PFd; $p < 0.05$). Dry matter and energy ADCs in PFd were lower than in FOd (75 vs 79% and 86 vs 88%, respectively), but similar to MFd ADC values. Total SFA digestibility was significantly lower ($p < 0.05$) in MFd than in the other diets (85 vs 91%). Total MUFA ADC was similar in all diets. In general, regardless of the fatty acid source, each individual fatty acid was well digested by European seabass, with ADC values ranging from 78 to 98%. Total PUFA ADC was similar between FOd and MFd, and was higher in those diets than in PFd (97 – 98 vs 96%). Total n-3 PUFA were better digested in FOd than in the other diets, while MFd had the highest total n-6 PUFA ADC. FOd showed significantly higher ADC values in important fatty acids such as EPA (20:5n-3), compared to PFd, and DHA (22:6n-3), compared to PFd and MFd. Alpha-linolenic acid (18:3n-3) was also better digested in FOd than in PFd, but no differences were observed between FOd and MFd.

Table 22. Nutrient apparent digestibility coefficients (ADC) of the experimental diets.

ADC (%)	FOd	PFd	MFd
DM	79.22 ± 0.61 ^{a}	74.79 ± 2.20 ^{b}	76.89 ± 1.92 ^{ab}
Lipid	90.49 ± 1.81	89.68 ± 2.58	87.84 ± 3.10
Protein	93.20 ± 0.21^{ab}	92.42 ± 0.47^{b}	93.36 ± 0.61^{a}
Energy	88.41 ± 0.36 ^{a}	86.13 ± 1.27 ^{b}	86.57 ± 1.08 ^{ab}
Fatty acids			
14:0	94.24 ± 1.19 ^{a}	93.99 ± 0.74 ^{a}	88.78 ± 1.53 ^{b}
16:0	91.14 ± 1.94 ^{a}	90.77 ± 0.98 ^{ab}	88.26 ± 1.20 ^{b}
18:0	88.16 ± 2.37 ^{ab}	92.77 ± 0.48 ^{a}	77.72 ± 1.93 ^{b}
16:1*n*-7	96.01 ± 0.60 ^{ab}	95.38 ± 0.89 ^{b}	96.90 ± 0.60 ^{a}
18:1*n*-9	95.59 ± 0.71	95.45 ± 0.83	94.80 ± 1.03
18:1*n*-7	94.65 ± 0.88 ^{a}	95.63 ± 0.75 ^{a}	90.01 ± 1.43 ^{b}
20:1*n*-9	94.19 ± 1.06 ^{a}	93.77 ± 0.93 ^{a}	91.49 ± 1.25 ^{b}
18:2*n*-6	96.67 ± 0.45 ^{ab}	95.58 ± 0.82 ^{b}	97.35 ± 0.56 ^{a}
18:3*n*-3	97.11 ± 0.47 ^{a}	95.75 ± 0.87 ^{b}	97.58 ± 0.46 ^{a}
20:5*n*-3	98.24 ± 0.08 ^{a}	96.35 ± 0.30 ^{b}	96.21 ± 0.65 ^{ab}
22:5*n*-3	97.90 ± 0.05 ^{a}	96.46 ± 0.21 ^{ab}	95.79 ± 0.70 ^{b}
22:6*n*-3	97.87 ± 0.11 ^{a}	94.73 ± 0.20 ^{b}	94.25 ± 1.34 ^{b}
∑ SFA [1]	90.97 ± 1.91 ^{a}	91.46 ± 0.82 ^{a}	85.35 ± 1.40 ^{b}
∑ MUFA [2]	95.05 ± 0.83	95.27 ± 0.84	94.44 ± 1.02
∑ PUFA [3]	97.64 ± 0.11 ^{a}	95.58 ± 0.74 ^{b}	97.00 ± 0.62 ^{a}
∑ *n*-3 [4]	97.95 ± 0.10 ^{a}	96.51 ± 0.34 ^{b}	96.14 ± 0.80 ^{b}
∑ *n*-6 [5]	96.69 ± 0.34 ^{ab}	95.52 ± 0.81 ^{b}	97.17 ± 0.59 ^{a}

Values are presented as mean (n = 4). Values in the same row with different superscript letter differ significantly ($p < 0.05$). STD, standard deviation.

[1] ∑ SFA is the sum of saturated fatty acids, which also includes 12:0, 13:0, 15:0, 17:0, 20:0, 21:0;
[2] ∑ MUFA is the sum of monounsaturated fatty acids, which also includes 14:1n-5, 17:1n-7, 22:1n-11;
[3] ∑ PUFA is the sum of polyunsaturated fatty acids, which also includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-6, 18:4n-1, 18:4n-3, 20:3n-6, 20:3n-3, 20:4n-3, 20:4n-6, 21:5n-3, 22:5n-6;
[4] ∑ n-3 is the sum of n-3 PUFA; [5] ∑ n-6 is the sum of n-6 PUFA.

Discussion

The rendered animal fats characterized in this study had distinct fatty acid profiles, reflecting the origin of each lipid source. The fish oil used in this study (FO), obtained from rendering by-products of fisheries and canning industries, had slightly lower amounts of total saturated fatty acids (SFA, 300 vs 340 g/kg), and higher total monounsaturated fatty acids (MUFA, 276 vs 250) and n-3 polyunsaturated fatty acids (PUFA, 320 vs 270 g/kg) than referenced anchovy fish oil (anchovy fish oil, 7-01-994; NRC (2011)). Although FO had lower EPA (20:5n-3) level than anchovy fish oil (85 vs 170 g/kg), it had higher level of DHA (22:6n-3; 191 vs 90 g/kg), suggesting that rendered FO is a valuable lipid source to use as fish oil replacement. The rendered fish oil used in this study mainly came from tuna fish, which has very high DHA levels (NRC, 2011). The fatty acid profiles of the two land animal fats tested (PF and MF) were in general agreement with values described in the literature for these fats (poultry fat, 4-09-319; beef tallow, 4-08-127; and pork lard, 4-04-790, NRC (2011)). The tested land animal fats had higher amounts of MUFA than FO (440 – 449 vs 276 g/kg) and lower amounts of n-3 PUFA (9 – 17 vs 320 g/kg), which is coherent with previous reports (Emery et al., 2014; Martins et al., 2009). Furthermore, similarly to what was described by Emery et al. (2014), the PF used in this study had a high amount of n-6 PUFA when compared to FO (252 vs 45 g/kg) due to its elevated content in linoleic acid (18:2n-6; 238 vs 20 g/kg). Although PF had also a lower amount of total n-3 PUFA, it had a higher amount of alpha-linolenic acid (18:3n-3; 15 vs 6 g/kg) compared to FO; since seeds are the main feed source for poultry and they are very rich in this fatty acid, this is probably a result of the poultry feeding habits. However, the lack of essential fatty acids such as EPA and DHA was clear in PF and MF, suggesting this could limit their use as single fat source in fish feeds. The amount of SFA in PF was similar to FO (279 vs 299 g/kg), but MF was richer in these fatty acids (411 g/kg), which was also consistent with the literature (Emery et al., 2014; Martins et al., 2009). The peroxide value was only determinable in FO (5.7 mEq O2/kg), indicating this animal fat is the most prone to oxidative rancidity (Gray, 1978). FO had higher acidity than MF and PF (79 vs 42 – 48 g/kg), which indicates a higher degree of hydrolysis of this fat source, since the acidity reflects the amount of free fatty acids (Alm, 2013). This higher acidity value is expectable since PUFA are more susceptible to hydrolysis and oxidation and in the present study fish oil was the fat with higher PUFA content. Furthermore, the amount of insoluble impurities in the tested animal fats was the highest in PF (7 g/kg), suggesting this was the least refined fat. MF had 0.2 g/kg insoluble impurities, which is below the maximum recommended level for bovine fats (1.5 g/kg) to prevent TSE occurrence (Woodgate & Van Der Veen, 2004).

To our best knowledge, the classical approach used to evaluate the digestibility of non-fat feedstuffs, where a reference diet is compared with diets that replace 300 g/kg of that diet

with the feedstuff to test (NRC, 2011), is not feasible when fats are being evaluated. This is mainly due to the extremely high fat content of the experimental diets that hinder extrusion process, and because non-purified diets have many other ingredients suppling lipids. To overcome these two bottlenecks, in this study we chose to totally replace the supplemental fish oil by each animal fat to be evaluated. The ADC of each experimental diet was then assessed and compared, and the differences obtained should be mainly due to the fat source, since the remaining composition was unaltered. All the test diets were well digested by European seabass, regardless of the lipid source, resulting in similar total lipid apparent digestibility coefficients (ADC). Dry matter, total lipids and energy ADC were lower in PFd than in FOd (75 vs 79% and 86 vs 88%), however, the ADC of the animal fats tested was still high regardless of their origin, suggesting these ingredients can be used as energy source for aquafeeds (Watanabe, 1989). The protein ADC was similar among FOd and the land animal fat diets, however, PFd had a lower protein ADC than MFd, in spite of diets being isonitrogeneous. Previous studies have also reported altered protein ADC related with the use of different lipid sources in fish (Martins et al., 2009) and in pigs (Jørgensen & Fernández, 2000). Since dietary lipids can change the composition of enterocyte cell membranes, they may affect their physiological functions (Jørgensen & Fernández, 2000), leading to different absorptive capabilities.

The individual fatty acid ADC results obtained in European seabass were generally high (78 – 98%) irrespectively of the fatty acid source. The ADC of total PUFA was similar between FOd and MFd, but PFd had a significantly lower digestibility value (96 vs 97 – 98%). Furthermore, even though PF had a higher amount of linoleic and alpha-linolenic acids than FO, the ADCs of these PUFA were lower in PFd than in FOd and MFd (96 vs 97 – 98%). In MFd, however, the ADCs of linoleic and alpha-linolenic acids were similar to FOd and resulted in similar total PUFA ADC values. These results are in agreement with a previous study by Martins et al. (2009) reporting that in Atlantic halibut PF had not only a lower total PUFA ADC value compared to herring oil (93 vs 97%) but also lower linoleic and alfa-linolenic ADC in PF. The position of fatty acids in triglycerides can have an important impact on fatty acids digestibility, as long-chain FA bound to the sn-1 and sn-3 positions are not absorbed as effectively as those in the sn-2 position in pigs (Kerr, Kellner, & Shurson, 2015). The lower absorption of 18:3n-3 and 18:2n-6 in poultry fat could be explained if these fatty acids were mainly bound to sn-1 or sn-3 positions, but not in the other animal fats. However, literature reports that, in chicken fat and fish oil, 18:2 are mainly bound to sn-2 positions while in pig fat they are mainly in sn-1 and sn-3 positions and in beef fat this fatty acid is evenly distributed in all three triglyceride positions, which is also the case for 18:3 in all these fats (Mottram, Crossman, & Evershed, 2001). It would be important to ascertain what causes the decrease

in the ADC of these fatty acids in poultry fat. In fish this is still controversial: while in trout pancreatic lipase hydrolyses unsaturated fatty acids in the sn-2 position, other fish species enzymes have shown 1,3-specificity (Tocher, 2003).

The present study showed that SFA ADC was lower in MFd than in FOd, which is consistent with previous results showing that SFA rich ingredients tend to have worse ADC values than those rich in MUFA or PUFA (Hua & Bureau, 2009). In fact, 16:0 and 18:0 are the most abundant SFA in MFd, and in lard, 16:0 is mainly bound at the sn-2 position of the glycerol molecule (Mu & Hoy, 2004), which should indicate a higher absorption efficiency. However, in the present study, even though MFd had both tallow and lard, the lipid source in this diet was mainly lard (700 g/kg) and this diet had the lowest 16:0 digestibility. High fatty acid intake usually produces higher apparent digestibility coefficients, since the relationship between endogenous FA production and dietary FA decreases (Smink, Gerrits, Hovenier, Geelen, Lobee, Verstegen, & Beynen, 2008). Nonetheless, the endogenous production of 16:0 and 18:0 might have increased in fish fed MFd, leading to a decreased apparent digestibility of those FA, as previously suggested in pigs Jørgensen and Fernández (2000) and in broiler chickens by Smink et al. (2008). The observed ADC values of SFA in the fish oil diet (91%) are lower than those previously reported for European seabass (95%) at 20 ºC (Eroldoğan, Yılmaz, Turchini, Arslan, Sirkecioğlu, Engin, Özşahinoğlu, & Mumoğullarında, 2013), but higher than values obtained for gilthead seabream (*Sparus aurata*; 83%) at 22 ºC (Trullàs, Fontanillas, Tres, & Sala, 2016) or for Atlantic halibut (83%) at 12 ºC (Martins et al., 2009). In salmonids, ADC values of SFA ranged from 47 to 86%, depending on the water temperature (1 – 12 ºC; Caballero, Obach, Rosenlund, Montero, Gisvold, and Izquierdo (2002); Menoyo, Lopez-Bote, Bautista, and Obach (2003)). It has been previously shown that the digestibility of SFA increases with the increase of temperature (Ng, Sigholt, & Bell, 2004), but observed differences among studies also seem to be related to the species ability to digest animal fats. As European seabass is eurythermic (5 – 28 °C; FAO (2017)) and may be reared in different temperature conditions, the ADC of animal fats should be further tested in a wider temperature range, including lower temperatures than the ones used in this study, and that are often reported during winter, as this could lead to lower digestibility coefficients and limit the dietary use of such fats during cold seasons.

EPA and DHA, essential fatty acids for fish growth, were better digested in the diet with FO than with other lipid sources (98 vs 94 – 96%). This shows that land animal fats not only lack these essential fatty acids, but that their fatty acid profile (rich in SFA and MUFA) seems to have impaired the digestibility of EPA and DHA present in these diets (provided by the dietary fishmeal). This was probably a result of the decreased PUFA/SFA ratio in PFd and MFd, which has been suggested to lead to a reduction in lipolytic activity and general reduction

in the fatty acids ADC (Caballero et al., 2002). Also, Menoyo et al. (2003) suggested that even though low levels of SFA may be easily digested, high dietary SFA levels (< 30% of the total fatty acids) can lead to impaired emulsion or the formation of micelles, compromising the digestibility of unsaturated fatty acids (Menoyo et al., 2003). The reduced EPA and DHA digestibility, combined with the lack of these fatty acids in land animal fats, indicates that their use as fish oil replacement could impact the final fillet nutritional value. In fact, a recent study by Monteiro, Matos, Ramos, Campos, and Valente (2018) showed that using a combination of poultry and mammal fat as fish oil replacement in seabass led to reduced EPA and DHA levels in muscle, even when only 25% fish oil was replaced.

The present results suggest that the different animal fats tested can all be considered feasible alternatives to fish oil as energy sources for European seabass. Using these animal fats, obtained from processing local by-products, could help decrease the dependency of imported fish oil for aquafeeds in EU and increase the sustainability of local slaughterhouses, contributing to a circular economy. Nevertheless, the inclusion of land animal fats resulted in lower digestibility coefficients of n-3 fatty acids. Growth trials using these land animal fats as fish oil replacements are warranted to evaluate the effects on growth performance and flesh quality induced by such replacements. Furthermore, as European seabass may be reared in different temperature conditions throughout the Mediterranean, the ADC of these animal fats should be tested in a wider temperature range, as the high amounts of saturated fatty acids in these animal fats could lead to lower digestibility coefficients under colder water temperatures (< 21 ºC), with impact on the use of such fats.

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Chapter V

Partial and total replacement of fish oil by poultry fat in diets for European seabass (*Dicentrarchus labrax*) juveniles

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Partial and total replacement of fish oil by poultry fat in diets for European seabass (*Dicentrarchus labrax*) juveniles: Effects on nutrient utilization, growth performance, tissue composition and lipid metabolism

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Partial and total replacement of fish oil by poultry fat in diets for European seabass (*Dicentrarchus labrax*) juveniles: effects on nutrient utilization, growth performance, tissue composition and lipid metabolism

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Abstract

Rendered fats can be sustainable alternatives to fish oil due to their lower price and wider availability. This study evaluates the effects of replacing fish oil by increasing levels of poultry fat (PF) on the nutrient utilization, growth performance, tissue composition and lipid metabolism of European seabass (*Dicentrarchus labrax*) juveniles. Homogeneous groups of 20 fish (19.1 ± 2.6 g) were subjected to a 12-h light/12-h dark photoperiod regime and kept in a recirculating saltwater system (salinity 35‰, 21 ± 1 ºC) for 16 weeks. Triplicate groups of fish were fed a commercial-based diet as control (FO) and four isonitrogenous experimental diets, with 25 (25PF), 50 (50PF), 75 (75PF) and 100% (100PF) fish oil replacement. Soy lecithin was also tested in the diet with 100% PF (100PFL) as an emulsifier agent. The inclusion of PF did not affect nutrient's digestibility nor plasma metabolites at 24 h post-feeding. Final body weight (62 – 67 g), feed intake, feed conversion ratio, protein efficiency ratio and whole-body composition were similar among experimental diets. Selected lipogenic enzymes' activities were similar among FO and PF diets. The fatty acids profile of muscle, liver and heart reflected the dietary lipid source, decreasing PUFA and increasing MUFA contents with PF inclusion. Fish fed 25PF and 50PF were still able to provide the EPA and DHA muscle levels recommended for human consumption, but 100PF resulted in increased liver lipid accumulation and reduced muscle omega-3 levels. Including soy lecithin could significantly attenuate liver lipid content in fish fed PF-based diets.

Introduction

The production costs of European seabass (*Dicentrarchus labrax*), a marine fish species with a high economic impact in Mediterranean countries, are closely related with aquafeed price, which comprise over 50% of the total costs of intensive aquaculture production (Rana et al., 2009). Any fluctuation on the feedstuffs price has, therefore, a relevant economic impact in the seabass feed and production costs, ultimately affecting seabass market price. While fish oil has been the main dietary source of essential fatty acids for marine carnivorous fish, the depletion of fishmeal stocks and increasing fish oil prices have made this ingredient environmentally and economically unsustainable. Fish oil is a major source of long-chain highly unsaturated fatty acids (HUFA), such as eicosapentaenoic (20:5*n*-3; EPA) and docosahexaenoic (22:6*n*-3; DHA) acids. These fatty acids are not only important to promote optimal growth and health of farmed marine fish (Sargent et al., 2002), but are also associated with the prevention of cardiovascular and inflammatory diseases (Ruxton et al., 2004), and the enhancement of brain development and mental health (Sargent, 1997) in humans. Since most available lipid alternatives have, at most, vestigial levels of EPA and DHA (Trushenski & Lochmann, 2009), replacing fish oil is a difficult task. The replacement of dietary fish oil may not only have an impact on the final tissue fatty acid composition, but also compromise fish metabolism and health, affecting fish growth performance and final product quality (Trushenski & Lochmann, 2009), and ultimately impacting human health (Sargent, 1997).

Rendered animal fats, obtained by processing animal by-products from slaughterhouses, can be sustainable alternatives to fish oil, due to their relatively low price and wide availability. There is a huge annual production of rendered fats in Europe (12 Mt; EPFRA (2016)) and their valorization could benefit both the companies that produce them and the aquafeed industry as end-user. Using rendered fats could significantly decrease the environmental impact associated with aquafeeds by reducing net primary production use (Papatryphon et al., 2004). Poultry fat (PF), obtained from rendering poultry by-products, represents a great percentage of the total rendered animal fats produced in Europe (EPFRA, 2016). The PF fat is richer in monounsaturated fatty acids (MUFA) and poorer in polyunsaturated fatty acids (PUFA) than fish oil (Emery et al., 2014), being particularly rich in *n*-6 PUFA and poor in *n*-3 PUFA. Although PF has a higher content of α-linolenic acid (18:3*n*-3) than fish oil (Emery et al., 2014), it lacks important *n*-3 HUFA, such as EPA and DHA. In face of EPA and DHA importance to marine fish, final quality of fish fillet and human health, the use of PF in aquafeeds might have to be cautioned. On the other hand, while the elevated levels of PUFA in fish oil make it prone to oxidation, PF, rich in MUFA, is more resistant to auto-oxidation, thus a more stable ingredient to be included in aquafeeds (Watanabe, 1989).

The replacement of fish oil by PF was reported to have no negative effect on growth performance and whole-body composition, except for a change in muscle fatty acid composition in Japanese sea bass (*Lateolabrax japonicus*), largemouth bass (*Micropterus salmoides*) and barramundi (*Lates calcarifer*; Ahmad et al. (2013); Xue et al. (2006); Yun et al. (2013)). Fish oil replacement by PF led to a higher amount of MUFA and a lower amount of total PUFA in fish muscle, in particular *n*-3 PUFA, and higher of *n*-6 PUFA (Ahmad et al., 2013; Bowyer et al., 2012; Friesen et al., 2015; Yun et al., 2013), reflecting PF fatty acid profile. However, according to Friesen et al. (2015), a finishing diet with fish oil could fully restore the fatty acid profile of the muscle in fish previously fed with PF.

Campos et al. (2016) have recently reported a high digestibility of rendered animal fats from poultry and mammal sources by European seabass juveniles. While PF reduced PUFA apparent digestibility coefficient (ADC), in particular *n*-3 PUFA ADC, compared to fish oil, total lipids, MUFA and saturated fatty acids (SFA) ADC values remained similar among diets (Campos et al., 2016). In Atlantic halibut (*Hippoglossus hippoglossus*), not only reduced PUFA ADC values were reported when PF replaced dietary fish oil, but also MUFA showed a lower digestibility (Martins et al., 2009). Since PF has high levels of MUFA, which have been reported to decrease lipid digestibility in fish (Hua & Bureau, 2009), the dietary inclusion of soy lecithin could help overcome this issue, as it was reported to enhance the activity of digestive enzymes in carp (*Cyprinus carpio*; Adel et al. (2017)).

To our knowledge, there is no information on the use of PF as direct fish oil replacer in European seabass diets. Moreover, promising results have recently evidenced the possibility of replacing up to 75% fish oil by a blend of PF and mammal fat without impairing seabass growth (Monteiro et al., 2018). Therefore, the present study was conducted to assess the effects of replacing fish oil by increasing levels of PF on the nutrient utilization, growth performance, tissue composition and lipid metabolism in European seabass (*D. labrax*) juveniles. The use of soy lecithin as an emulsifier agent was also evaluated.

Materials and methods

The present study was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

Ingredient origin and experimental diets

Table 23. Composition and fatty acid profiles of the lipid sources used.

Chemical composition	Fish oil	Poultry fat
Dry matter (DM, %)	99.7	100.0
Total lipid (% DM)	88.5	86.8
Gross energy (kJ g^{-1} DM)	39.6	40.0
Acidity (%)	7.9	4.8
Peroxide value (mEq O_2 kg^{-1})	5.7	ND[f]
Insoluble impurities (%)	0.2	0.7
Fatty acids (g 100 g^{-1} total fatty acids)		
14:0	3.9	0.5
16:0	17.7	21.3
18:0	4.9	5.7
∑ SFA[a]	30.0	27.9
16:1*n*-7	5.4	4.9
18:1*n*-9	14.8	36.7
18:1*n*-7	2.6	1.8
20:1*n*-9	1.9	0.2
∑ MUFA[b]	27.6	44.0
18:2*n*-6	2.0	23.8
18:3*n*-3	0.6	1.5
20:5*n*-3	8.5	0.0
22:5*n*-3	1.7	0.0
22:6*n*-3	19.1	0.0
∑ PUFA[c]	37.6	23.0
∑ PUFA*n*-6 [d]	4.5	25.2
∑ PUFA*n*-3 [e]	32.0	1.7
∑ *n*-3/ ∑ *n*-6	7.1	0.1

[a] ∑ SFA, sum of saturated fatty acids, includes 12:0, 13:0, 14:0 15:0, 16:0 17:0, 18:0, 20:0, 21:0;
[b] ∑ MUFA, sum of monounsaturated fatty acids, includes 16:1*n*-9, 14:1*n*-5, 17:1*n*-7, 18:1*n*-9, 18:1*n*-7, 22:1*n*-11;
[c] ∑ PUFA, sum of polyunsaturated fatty acids, includes 16:2*n*-4, 16:3*n*-4, 16:4*n*-1, 18:2*n*-6, 18:2*n*-4, 18:3*n*-3, 18:3*n*-6, 18:4*n*-1, 18:4*n*-3, 20:3*n*-6, 20:3*n*-3, 20:4*n*-3, 20:4*n*-6, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:5*n*-6, 22:6*n*-3;
[d] ∑ PUFA*n*-6, sum of *n*-6 PUFA, includes 18:2*n*-6, 18:3*n*-6, 20:3*n*-6, 20:4*n*-6, 22:5*n*-6;
[e] ∑ PUFA*n*-3, sum of *n*-3 PUFA, includes 18:3*n*-3, 18:4*n*-3, 20:3*n*-3, 20:4*n*-3, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:6*n*-3;
[f] ND, Non detected or bellow detection level.

The selected PF was supplied by SAVINOR S.A. (Portugal) and obtained by rendering poultry by-products from slaughterhouses in Portugal. A commercial-based diet, with fish oil supplementation, was used as control (FO), and four experimental diets were formulated with 25, 50, 75 and 100% fish oil replacement by PF (25PF, 50PF, 75PF and 100PF, respectively). An additional diet with 100% fish oil replacement by PF and with the inclusion of soy lecithin

(at 1%) was formulated (100PFL). The fatty acids and analytical composition of each lipid source (fish oil and PF) are presented in Table 23. All diets were isoenergetic (23 kJ g^{-1} dry matter, DM) and isonitrogenous (51% crude protein, DM basis; Table 24). Diets' formulation and proximate composition are shown in Table 24 and the dietary fatty acids profile is presented in Table 25. Diets were manufactured by SPAROS, Lda. (Portugal), by means of a pilot-scale twin-screw extruder (CLEXTRAL BC45, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110 °C. Pellet size was 2.5 mm. Upon extrusion, all batches were dried in a convection oven (OP 750-UF, LTE Scientifics, UK) for 3 h at 60 °C and allowed to cool at room temperature. Supplemented lipids were subsequently added to each diet under vacuum in a Pegasus vacuum coater (PG-10VCLAB, Dinnisen, The Netherlands).

Table 24. Formulation and proximate composition of the experimental diets.

Ingredients (%)	FO	25PF	50PF	75PF	100PF	100PFL
Fishmeal 60 (National)[a]	22.0	22.0	22.0	22.0	22.0	22.0
CPSP 90[b]	3.0	3.0	3.0	3.0	3.0	3.0
Feather meal[c]	5.0	5.0	5.0	5.0	5.0	5.0
Haemoglobin powder[d]	5.0	5.0	5.0	5.0	5.0	5.0
Poultry meal[e]	20.0	20.0	20.0	20.0	20.0	20.0
Pea protein concentrate[f]	3.5	3.5	3.5	3.5	3.5	3.5
Soybean meal 48[g]	9.0	9.0	9.0	9.0	9.0	9.0
Rapeseed meal[h]	3.5	3.5	3.5	3.5	3.5	3.5
Wheat meal[i]	14.8	14.8	14.8	14.8	14.8	13.8
Fish oil[j]	12.0	9.0	6.0	3.0	0.0	0.0
Poultry fat[k]	0.0	3.0	6.0	9.0	12.0	12.0
Vit & Min Premix PV01[l]	1.0	1.0	1.0	1.0	1.0	1.0
Lutavit E50[m]	0.03	0.03	0.03	0.03	0.03	0.03
Choline chloride[n]	0.07	0.07	0.07	0.07	0.07	0.07
Betaine HCl[o]	0.1	0.1	0.1	0.1	0.1	0.1
Soy lecithin – Powder[p]	0.0	0.0	0.0	0.0	0.0	1.0
Binder (Kieselghur)[q]	0.3	0.3	0.3	0.3	0.3	0.3
Antioxidant[r]	0.2	0.2	0.2	0.2	0.2	0.2
Sodium propionate[s]	0.1	0.1	0.1	0.1	0.1	0.1
L-Lysine[t]	0.2	0.2	0.2	0.2	0.2	0.2
DL-Methionine[u]	0.1	0.1	0.1	0.1	0.1	0.1
L-Taurine[v]	0.1	0.1	0.1	0.1	0.1	0.1
Chemical composition						
Dry matter (DM, %)	94.4	92.2	92.9	93.8	93.0	93.5
Crude protein (% DM)	50.8	52.9	51.6	51.7	51.1	51.8
Crude fat (% DM)	18.7	18.9	18.4	19.6	19.2	20.2
Gross energy (kJ/g DM)	22.8	23.2	23.1	23.3	23.2	23.3
Ash (% DM)	10.6	10.9	10.9	10.6	10.6	10.9
Total P (% DM)	1.4	1.4	1.3	1.3	1.3	1.5

The abbreviations for the experimental diets stand for: FO – reference diet; 25PF, 50PF, 75PF, 100PF and 100PFL – diets with 25, 50, 75, 100% fish oil replacement with poultry fat and 100% replacement plus soy lecithin, respectively;

[a] Fair Average Quality (FAQ) fishmeal: 62% CP, 12% CF, COFACO, Portugal;

[b] CPSP90: 84% CP, 12% CF, SOPROPÊCHE, France;

[c] Feather meal: 84.2% CP, 10.4% CF, AVICASAL SA, Portugal;

[d] Hemoglobin powder: 91.6% CP, 1.2% CF, SONAC BV, The Netherlands;

[e] Poultry by-product meal: 69,1% CP, 13,7% CF, SAVINOR SA, Portugal;

[f] NUTRALYS F85F: 78% CP, 1% CF, ROQUETTE Frères, France;

[g] Dehulled solvent extracted soybean meal: 47.7% CP, 2.2% CF, Cargill, Spain;

[h] Rapeseed meal: 36% CP, 2.7% CF, PREMIX Lda, Portugal;

[i] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal;
[j] Fish oil, SAVINOR SA, Portugal;
[k] Poultry fat, SAVINOR SA, Portugal;
[l] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg kg $^{-1}$ diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings;
[m] Lutavit E50: Premix Lda, Portugal;
[n] Choline chloride: PREMIX Lda, Portugal;
[o] Betaine HCl: PREMIX Lda, Portugal;
[p] Soy lecithin powder: Lecico P700IPM, LECICO GmbH, Germany;
[q] Kielseguhr (natural zeolite): LIGRANA GmbH, Germany;
[r] Antioxidant powder, Paramega PX, Kemin Europe NV, Belgium;
[s] Sodium propionate, Premix Lda, Portugal;
[t] L-Lisine HCl 99%: Ajinomoto Eurolysine SAS, France;
[u] DL-Metionine 99%: Evonik Degussa GmbH, Germany;
[v] L-Taurine: PREMIX Lda, Portugal.

Growth trial

The growth trial was conducted in the Fish Culture Experimental Unit of CIIMAR (Portugal), with juvenile European seabass obtained from a commercial fish farm (Maresa, Mariscos de Estero S.A., Ayamonte, Spain). To adapt to the experimental conditions, fish were kept in quarantine for 4 weeks and fed a commercial diet (AQUASOJA, Sorgal S.A., Portugal; 49% crude protein and 20% crude fat, DM basis).

After acclimatization, fish were individually weighed (g) and measured (total length, cm) and 18 homogeneous groups of 20 fish (mean body weight 19.1 ± 2.6 g, mean body length 12.5 ± 0.6 cm) were randomly distributed by 55 L fiberglass tanks within a saltwater recirculation system. Also, 15 fish from the initial fish stock were collected after a 24 h fasting period, sacrificed by anaesthetic overdose (MS222, 150 mg L^{-1}), and then kept at −20 ºC, until initial whole-body composition was analyzed. Fish were adapted to the new conditions for 2 days (water temperature of 21 ± 1 ºC, salinity of 35 ‰, flow rate at 2 L min^{-1} and 12 h light/12 h dark photoperiod regime). Each diet was randomly assigned to triplicate groups of fish that were fed until apparent satiation, three times a day, by automatic feeders. The amount of feed supplied to each tank was adjusted daily based on the presence or absence of uneaten feed in each tank (Campos et al., 2017). Throughout the 16 weeks of the trial, nitrogenous compounds and pH were monitored and kept at levels recommended for marine species. An intermediate sampling was conducted at week 8, in which fish were bulk weighed to monitor weight gain and register feed consumption.

At the end of the 16-week period, each fish was individually weighed (g) and measured (total length, cm) after a 24-hour fasting period. Blood was collected from the caudal vein of six fish per dietary treatment, at 2, 6, 12 and 24 h post-feeding, using syringes with EDTA. Samples were centrifuged (5000 *g* for 10 min at 4 °C) and the resulting plasma was stored at −80 °C for metabolite analysis. To minimize fish stress due to handling, at each sampling time blood was collected from six fish of one tank per dietary treatment, allowing each tank to be only sampled again after at least 12 hours. At 24 hours post-feeding, blood was collected from five fish of every tank. These five fish were then sacrificed by a sharp blow on the head prior to sampling the dorsal and ventral muscle, which were immediately frozen in liquid nitrogen and kept at −80 ºC until determination of total lipids and fatty acids profile. Dorsal and ventral muscle were collected and analyzed separately. Heart, viscera and liver from these fish were also sampled, weighed and frozen in liquid nitrogen and kept at −80 ºC, for total lipids and fatty acids determination (viscera were only analyzed for their total lipid content) and for analysis of selected lipogenic enzymes activity in liver. Before being placed in liquid nitrogen, a portion of each liver was cut and fixed in phosphate-buffered formalin 4% (pH 7) for 24 hours and kept in alcohol 70% until further processing for histological evaluation. Another five fish per tank were collected, sacrificed by anaesthetic overdose (MS222, 150 mg L^{-1}) and frozen at −20 ºC until whole body composition analysis. The remaining fish were used to evaluate the *in vivo* digestibility of the experimental diets.

Table 25. Fatty acids profile of the experimental diets (g $100g^{-1}$ total fatty acids).

Fatty acids	FO	25PF	50PF	75PF	100PF	100PFL
14:0	3.3	3.1	2.4	2.1	1.2	1.2
16:0	18.0	19.2	19.6	20.5	20.2	20.2
18:0	5.1	5.2	5.3	5.5	5.7	5.6
∑ SFA[a]	29.0	29.7	29.3	29.8	28.1	28.0
16:1*n*-7	4.7	4.7	4.7	4.0	4.6	4.5
18:1*n*-9	17.8	21.2	25.2	29.0	32.9	32.4
18:1*n*-7	2.5	2.5	2.4	2.6	2.1	2.1
20:1*n*-9	2.2	1.9	1.6	1.5	1.1	1.1
∑ MUFA[b]	30.5	33.5	36.4	39.3	42.5	41.9
18:2*n*-6	7.4	10.9	14.3	14.4	20.7	21.4
18:3*n*-3	1.1	1.3	1.4	1.3	1.6	1.6
20:4*n*-6	1.5	1.1	1.0	0.8	0.7	0.7
20:5*n*-3	6.3	5.4	3.9	2.4	1.0	1.0
22:5*n*-3	1.4	1.0	0.7	0.5	0.3	0.3
22:6*n*-3	13.8	9.7	6.9	4.3	1.8	1.8
∑ PUFA[c]	36.3	33.1	31.2	26.1	27.5	28.3
∑ PUFA*n*-6[d]	10.4	13.0	16.2	16.0	22.1	22.8
∑ PUFA*n*-3[e]	24.6	19.1	14.3	9.6	5.2	5.3
∑ *n*-3/ ∑ *n*-6	2.4	1.5	0.9	0.6	0.2	0.2

[a] ∑ SFA, sum of saturated fatty acids, includes 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0;
[b] ∑ MUFA, sum of monounsaturated fatty acids, includes 16:1*n*-9, 14:1*n*-5, 17:1*n*-7, 18:1*n*-9, 18:1*n*-7, 22:1*n*-11;
[c] ∑ PUFA, sum of polyunsaturated fatty acids, includes 16:2*n*-4, 16:3*n*-4, 16:4*n*-1, 18:2*n*-6, 18:2*n*-4, 18:3*n*-3, 18:3*n*-6, 18:4*n*-1, 18:4*n*-3, 20:3*n*-6, 20:3*n*-3, 20:4*n*-3, 20:4*n*-6, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:5*n*-6, 22:6*n*-3;

[d] ∑ PUFA*n*-6, sum of *n*-6 PUFA, includes 18:2*n*-6, 18:3*n*-6, 20:3*n*-6, 20:4*n*-6, 22:5*n*-6;
[e] ∑ PUFA*n*-3, sum of *n*-3 PUFA, includes 18:3*n*-3, 18:4*n*-3, 20:3*n*-3, 20:4*n*-3, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:6*n*-3.

Digestibility trial

After the growth trial, the remaining fish from each experimental dietary treatment were distributed into two new homogeneous groups (mean body weight 64 ± 12 g), randomly distributed by 55 L tanks with individual faeces sedimentation columns, in a system specially designed for digestibility studies (Guelph system) according to Cho & Slinger (1979). The ADCs of the experimental diets were determined by the indirect method, after the incorporation of 1% chromium oxide, as inert marker, to each diet. Each extruded diet used in the growth trial was ground and mixed with the marker before being dry pelleted through a 3.2 mm die at 50 ºC using a laboratory pellet press (CPM, C-300 model, S. Francisco, USA). Fish were subjected to the same rearing conditions and diets used during the growth trial. The marked diets (with chromium oxide) were supplied until apparent satiation, once a day, for 5 days, to adapt the fish before the faeces collection began. About 30 minutes after feeding, each tank was carefully cleaned to assure the absence of uneaten feed left in the tanks and in the sedimentation column. Faeces were collected every morning before feeding, from the sedimentation column, centrifuged to eliminate excess water and frozen at −20 ºC. Daily collection of faeces was performed for each experimental diet until obtaining an amount considered enough for chemical analysis (8 – 10 days). At the end of the trial, faeces were freeze-dried prior to analysis.

Proximate analysis of feed, whole body and faeces

Fish collected from each tank were ground and pooled. A sample was collected to determine the moisture content (105 ºC for 24 h), and the remaining was freeze-dried, ground and homogenized for proximate analysis. The experimental diets and freeze-dried faeces were also ground and homogenized prior to analysis (faeces were sifted). PF was liquefied (at about 35 ºC) and homogenized before being analysed. Acidity, peroxide value and insoluble impurities were determined in the tested lipid sources according to EN ISO 660:2002, ISO 3960:2007 and EN ISO 663:2005, respectively. All chemical analysis followed AOAC (2006) methods and were performed in duplicate. The lipid sources, diets, fish carcass and faeces were analysed for dry matter (105 ºC for 24 h) and gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). In the experimental diets, carcass and collected faeces, crude protein was determined (N x 6.25) using a Leco nitrogen analyser (Model FP-528, Leco Corporation, St. Joseph, USA), crude fat by petroleum ether extraction using Soxtec 2055 (Foss, Denmark) and ash content was determined by combustion in a

muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 500 ºC for 5 h). In the experimental diets and faeces, chromic oxide content was determined according to Bolin et al. (1952) and phosphorous content was determined by digestion at 230 ºC in a Kjeldatherm block digestion unit followed by digestion at 60 ºC in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406).

Liver histology

After fixation, liver samples were dehydrated, embedded in paraffin, sliced at 3 µm, and then stained with either hematoxylin and eosin (H&E) or periodic acid schiff (PAS). The H&E sections were examined to determine cytoplasm vacuolization degree, using a semi-quantitative approach. The following degrees and criteria were used, according to Figueiredo-Silva et al. (2005): Grade 1 (low) – about < 1/3 of the hepatocyte cytoplasm showed vacuolization; Grade 2 (moderate) – 1/3 < x < 2/3 of the hepatocyte cytoplasm showed vacuolization; Grade 3 (high) – > 2/3 of the hepatocyte cytoplasm showed vacuolization. PAS sections were used to determine the hepatocellular glycogen content, also using a semi-quantitative approach, based on the following degrees, according to Matos et al. (2013): Grade 1 (low) – hepatocyte cells in PAS sections presented a weak coloration; Grade 2 (mild) – PAS sections presented a mild coloration; Grade 3 (intense) – PAS sections presented a strong coloration. Both assessments were done by examining the stained sections by light microscopy (Olympus BX51, GmbH, Hamburg, Germany), at low resolution (× 10) for general aspects and at higher magnification (× 40) for grading. In each section, 20 randomly chosen sample fields were analysed.

Total lipids and fatty acids analysis

Total lipids of the dorsal and ventral muscle, liver, heart and viscera were extracted and quantified gravimetrically by the method described by Folch et al. (1957) modified by using dichloromethane instead of chloroform. The fatty acids in the lipid extracts of liver and heart were transesterified by acidic methylation to fatty acid methyl esters (FAME; Campos et al. (2017)). The FAME in the experimental diets, dorsal and ventral muscle were obtained using the same procedure but by direct transesterification. Nonadecanoic acid (19:0) was added as internal standard in all samples. FAME were then analyzed using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Europe GmbH, Germany), equipped with a flame-ionization detector (GC-FID) and a Omegawax 250 capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Supelco, Bellefonte, USA) and identified by comparison with known standard mixtures as described by Campos et al. (2017). FAME contents in ingredients, feed and tissues

were expressed as % of total FAME and as mg g^{-1} of the analysed sample, calculated using the internal standard added to each sample and lipid extract.

Plasma metabolites analysis

Plasma triglycerides, glucose, total cholesterol and non-esterified fatty acids (NEFA) levels were determined enzymatically using commercial kits (1001312, 1001190 and 1001090, Spinreact, Barcelona, Spain, and 434-91795 NEFA-HR (2) R1 and 436-91995 NEFA-HR (2) R2, Wako Chemicals, Neuss, Germany, respectively), adapting manufacturer's instructions to a microplate format.

Lipogenic enzyme activity measurements

The activities of selected lipogenesis enzymes were assessed on the supernatant of frozen liver samples, after being homogenized in six volumes of ice-cold buffer (100 mM Tris-HCL, 0.1 M EDTA, 0.1% (v v^{-1}) Triton X-100, 50 mM PMSF) and centrifuged at 30000 *g*, at 4 °C for 30 minutes. Glucose-6-phosphate dehydrogenase (G6PD) activity was analysed as described by Bautista et al. (1988), using a reaction mixture containing 1 M Tris–HCl buffer (pH 7.8), 200 mM $MgCl_2$, 10 mM NADP and 20 mM glucose-6-phosphate as substrate. Malic enzyme (ME) activity was analysed according to Ochoa (1955), using a reaction mixture containing 74.5 mM imidazole–HCl buffer (pH 7.4), 100 mM $MgCl_2$, 8 mM NADP and 40 mM L-malate as substrate. Fatty acid synthase (FAS) activity was assayed as described by Chang et al. (1967), modified by Chakrabarty & Leveille (1969) using a reaction mixture of 100 mM K_3PO_4 buffer (pH 6.5), 0.1 mM NADPH and 25 µM acetyl-CoA and 600 mM malonyl-CoA. Briefly, to determine all the above mentioned enzyme activities, assays were performed at 37 °C and the production or consumption of NADPH were monitored by changes in absorbance (at 240 nm), using a microplate reader (ELx808; Bio-Tek Instruments, Winooski, Vermont, USA). One unit of enzyme activity was defined as the amount of enzyme necessary to convert one µmol of substrate at assay temperature. All enzyme activities were expressed in milliunits per mg of hepatic soluble protein (specific activity) and, to do so, protein concentration was determined in each liver sample according to Bradford (1976), using bovine serum albumin as standard.

Calculations

The ADCs of the experimental diets were calculated according to Maynard et al. (1979): dry matter ADC (%) = 100 × [1− (dietary Cr_2O_3 level/faeces Cr_2O_3 level)] and nutrients or energy ADC (%) = 100 × [1− (dietary Cr_2O_3 level/faecesCr_2O_3 level) × (faeces nutrient or energy level／dietary nutrient or energy)]. Average body weight (ABW) = (final body weight +

initial body weight)/2. Digestible N, P, lipids (L) or energy (E) intake = (dry feed consumption × N, P, L (%) or E (kJ g^{-1}) in the diet × ADC N, P, L or E)/ABW/days. N, P, L or E gain = (final carcass N, P, L or E content – initial carcass N, P, L or E content)/ABW/days. N, P, L or E retention efficiency (NRE, PRE, LRE, or ERE) = (N, P, L or E gain/Digestible N, P, L or E intake) × 100. Fecal N, P, L or E losses = crude N, P, L or E intake × [1 − (ADC N, P, L or E/100)]. Non-fecal N, P or L losses = crude N, P or L intake − N, P or L gain − Fecal N, P or L losses. Non-fecal E losses = non-fecal N losses × 25 kJ N^{-1}. Metabolizable energy (ME) = digestible energy intake − non-fecal E losses. Total heat loss = ME − energy gain. K, Condition factor = [Final body weight/(Final body length)^3] × 100. Daily growth index (DGI) = 100 × [(Final body weight)$^{1/3}$ − (Initial body weight)$^{1/3}$]/days. Voluntary feed intake (VFI) = 100 × dry feed intake/average body weight/day. Feed conversion ratio (FCR) = dry feed intake/weight gain. Protein efficiency ratio = weight gain/crude protein intake. Hepatosomatic index (HSI) = 100 × liver weight/body weight. Viscerosomatic index (VSI) = 100 × weight of viscera/body weight. Heart weight to total body weight ratio = 100 × [heart weight (g)/final body weight (g)]. Atherogenicity index (AI) = [12:0 + (4×14:0) + 16:0]/(Σ PUFA*n*-6 + Σ PUFA*n*-3 + Σ MUFA). Thrombogenicity index (TI) = (14:0 + 16:0 + 18:0)/[0.5 × Σ MUFA + 0.5 × Σ PUFA*n*-6 + 3× Σ PUFA*n*-3 + (Σ PUFA*n*-3/Σ PUFA*n*-6)]. Hypocholesterolemic/hypercholesterolemic fatty acids ratio (h/H) = (18:1*n*-9 + 18:2*n*-6 + 20:4*n*-6 + 18:3*n*-3 + 20:5*n*-3+ 22:5*n*-3 + 22:6*n*-3)/(14:0 + 16:0). Peroxidation index (PI) =0.025 × (% Σ monoenoic fatty acids) + 1 × (% Σ dienoic fatty acids) + 2 × (% Σ trienoic fatty acids) + 4 × (% Σ tetraenoic fatty acids) + 6 × (% Σ pentaenoic fatty acids) + 8 × (% Σ hexaenoic fatty acids).

Statistical analysis

Data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and transformed whenever required before being submitted to a one-way ANOVA with the statistical program IBM SPSS STATISTICS, 22.0 package, IBM corporation, New York, USA (2011). When one-way ANOVA showed significance, individual means were compared using HSD Tukey test. If data did not meet the ANOVA assumptions, the non-parametric Kruskal-Wallis test was used. The Mann-Whitney test was used for individual means comparison when significance was observed. Student's T test was used to compare results obtained with 100PF and 100PFL diets. Data obtained from plasma metabolite assays were tested with a two-way ANOVA, with diets and sampling times as main effects. In all cases, significant differences were considered when $p < 0.05$.

Results

The fatty acids profile of the lipid sources used (fish oil and PF) are portrayed in Table 23. PF had a higher linoleic acid (18:2*n*-6) content than fish oil (24 *vs* 2%) but lacked HUFA like EPA (20:5*n*-3), and DHA (22:6*n*-3). Total MUFA levels were higher in PF mainly due to its high content in oleic acid (18:1*n*-9; 37 *vs* 15% in fish oil), while the amount of total SFA were similar between lipid sources. Fish oil had a higher acidity than PF (7.9 *vs* 4.8%). The peroxide value is only reported for fish oil as it was bellow detection level in PF. The insoluble impurities were higher in PF than in fish oil (0.70 *vs* 0.18).

All experimental diets were isolipidic and isonitrogenous (Table 24) and their fatty acids profile reflected the replacement of fish oil by PF (Table 25), leading to an overall increase of total MUFA and to a reduction of total PUFA, with a decrease in *n*-3 PUFA and an increase in *n*-6 PUFA. All experimental diets were well digested by European seabass (dry matter ADC > 74%) and the ADC of different nutrients and energy were similar among experimental diets (Table 16). Lipid gain was not affected by the experimental diets, although lipid fecal losses significantly decreased with increasing fish oil replacement up to 75% (Table 16). N and energy gain were also not affected by the experimental diets, even though the fecal losses were lowest in 75PF diet, compared with 100PF. The nutrients and digestible energy intake and the nutrient and energy retention efficiency (% digestible intake) were also not affected by the fish oil replacement (Table 16).

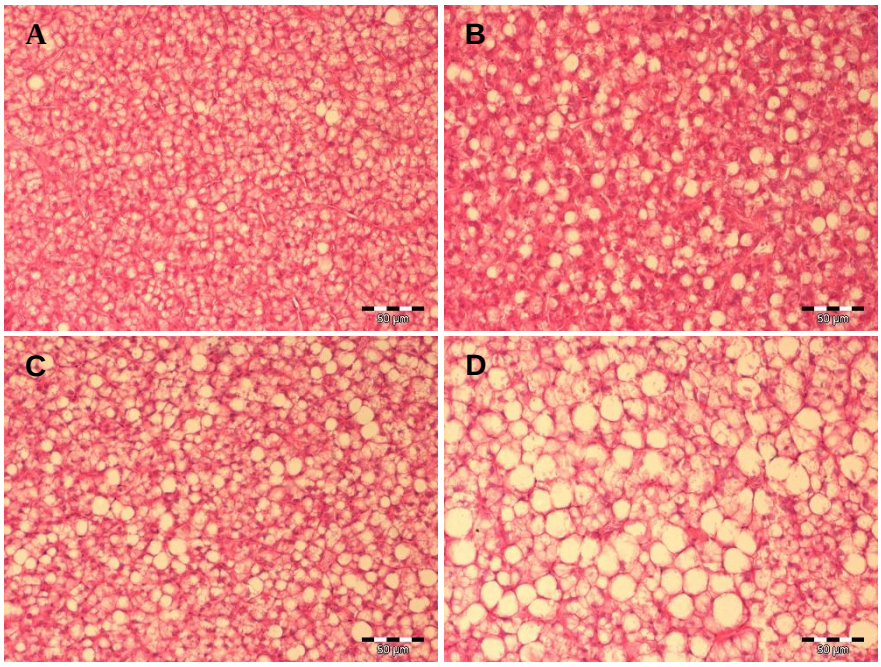

Figure 12. Liver sections of seabass fed FO (A), 25PF (B), 75PF (C) and 100PF (D) (H & E, 40×). High vacuolization of the hepatocyte cytoplasm is observed for fish fed PF-based diet (D) compared to fish fed FO diet (A).

Table 26. Apparent digestibility coefficients (ADC) and nutrient balances of the experimental diets.

ADC (%)	FO	25PF	50PF	75PF	100PF	100PFL
Dry matter	75.4 ± 0.5	75.7 ± 0.7	75.9 ± 0.8	76.8 ± 0.4	74.3 ± 1.9	74.9 ± 0.1
Protein	89.6 ± 0.1	89.9 ± 0.2	89.0 ± 0.9	90.1 ± 0.9	88.2 ± 1.1	88.8 ± 0.6
Energy	84.4 ± 0.4	84.8 ± 0.2	84.5 ± 0.5	85.7 ± 0.6	84.4 ± 0.1	84.7 ± 0.1
Lipids	93.8 ± 0.7	94.8 ± 0.5	94.7 ± 0.2	95.6 ± 0.4	94.1 ± 1.8	94.5 ± 0.4
Phosphorus	64.6 ± 1.5	60.3 ± 4.3	62.8 ± 1.3	63.0 ± 0.3	60.6 ± 0.1	56.8 ± 1.9
Nitrogen (N) balance (mg 100g ABW†$^{-1}$ day^{-1})						
Digestible N intake (DN)	100.0 ± 5.3	103.3 ± 5.2	99.5 ± 6.2	95.4 ± 5.0	100.3 ± 6.5	99.0 ± 1.1
N gain	28.3 ± 2.0	28.1 ± 0.7	28.8 ± 1.8	28.4 ± 1.5	26.9 ± 1.7	27.8 ± 1.2
NRE[1] (% DN)	30.7 ± 4.1	32.5 ± 2.5	31.1 ± 3.0	31.9 ± 1.8	26.9 ± 2.7	28.0 ± 0.9
Faecal N losses	11.6 ± 0.6^{ab}	11.6 ± 0.6^{ab}	12.3 ± 0.8^{ab}	10.5 ± 0.5^{b}	13.4 ± 0.9^{a}	12.5 ± 0.1
Non-faecal N losses	71.7 ± 5.5	75.3 ± 5.3	70.7 ± 6.5	67.0 ± 3.5	73.4 ± 7.1	71.2 ± 0.3
Lipid (L) balance (g kg ABW†$^{-1}$ day^{-1})						
Digestible L intake (DL)	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	2.5 ± 0.2	2.6 ± 0.03
L gain	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.1 ± 0.03	1.1 ± 0.2	1.3 ± 0.1
LRE[2] (% DL)	46.8 ± 6.8	50.5 ± 3.2	52.9 ± 3.8	47.7 ± 3.5	43.5 ± 8.8	48.8 ± 4.2
Faecal L losses	0.16 ± 0.008^{a}	0.13 ± 0.007^{b}	0.13 ± 0.008^{bc}	0.11 ± 0.006^{c}	0.16 ± 0.010^{a}	0.15 ± 0.002
Non-faecal L losses	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.03	1.3 ± 0.2	1.4 ± 0.3	1.3 ± 0.1
Energy (E) balance (kJ kg ABW†$^{-1}$ day^{-1})						
Digestible E intake (DE)	264.1 ± 14.0	266.5 ± 13.4	264.7 ± 16.4	255.2 ± 13.3	272.7 ± 17.6	265.6 ± 3.1
E gain	80.8 ± 8.7	86.4 ± 5.9	82.35 ± 11.6	81.24 ± 0.83	77.7 ± 9.33	88.0 ± 6.6
ERE[3] (% DE)	30.7 ± 4.1	32.5 ± 2.5	31.1 ± 3.0	31.9 ± 1.8	28.6 ± 4.43	33.1 ± 2.1
Faecal E losses	48.7 ± 2.6^{ab}	47.6 ± 2.4^{ab}	48.5 ± 3.0^{ab}	42.7 ± 2.2^{b}	50.4 ± 3.3^{a}	48.2 ± 0.6
Non-faecal E losses	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.2	1.7 ± 0.1	1.8 ± 0.2	1.8 ± 0.1
Metabolizable E	262.3 ± 13.9	264.6 ± 13.3	262.9 ± 16.2	253.5 ± 13.2	270.8 ± 17.4	263.8 ± 3.1
Total heat loss	183.2 ± 18.3	180.1 ± 13.9	182.3 ± 9.7	173.9 ± 13.6	194.9 ± 23.1	177.6 ± 3.6

Values are presented as mean ± standard deviation (n = 2 for determination of nutrient ADC; n = 3 for determination of remaining parameters). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). The presence of an * means significant differences between treatments 100PF and 100PFL ($p < 0.05$). Absence of superscript or absence of * indicates no significant difference between treatments.

† ABW, Average body weight;

[1] NRE, Nitrogen retention efficiency; [2] LRE, Lipid retention efficiency; [3] ERE, Energy retention efficiency.

Table 27. Growth performance, somatic indexes, final whole-body composition (% or kJ g $^{-1}$ of wet weight, WW), and total lipid content (% WW) of different organs of European seabass fed the experimental diets for 16 weeks.

Growth	FO	25PF	50PF	75PF	100PF	100PFL
Initial body weight (g)	19.1 ± 2.7	19.1 ± 2.6	19.1 ± 2.6	19.1 ± 2.5	19.1 ± 2.7	19.1 ± 2.8
Final body weight (g)	62.4 ± 10.3^{ab}	64.9 ± 10.5^{ab}	67.3 ± 11.1^{a}	62.7 ± 9.6^{ab}	61.5 ± 10.6^{b}	62.6 ± 13.2
Initial body length (cm)	12.5 ± 0.6	12.6 ± 0.6	12.5 ± 0.6	12.5 ± 0.6	12.5 ± 0.6	12.6 ± 0.6
Final body length (cm)	17.9 ± 1.1^{ab}	18.4 ± 1.1^{ab}	18.4 ± 1.0^{a}	18.1 ± 0.9^{ab}	17.9 ± 1.0^{b}	18.1 ± 1.1
Final K[1]	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
DGI[2]	1.2 ± 0.2^{ab}	1.2 ± 0.2^{ab}	1.3 ± 0.2^{a}	1.2 ± 0.2^{ab}	1.2 ± 0.2^{b}	1.2 ± 0.3
VFI[3]	1.4 ± 0.04	1.4 ± 0.07	1.3 ± 0.06	1.4 ± 0.06	1.4 ± 0.11	1.4 ± 0.07
FCR[4]	1.4 ± 0.09	1.3 ± 0.08	1.3 ± 0.10	1.3 ± 0.04	1.5 ± 0.21	1.4 ± 0.05
PER[5]	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.2	1.3 ± 0.1
HSI[6]	1.1 ± 0.1^{b}	1.1 ± 0.2^{b}	1.2 ± 0.2^{b}	1.2 ± 0.2^{b}	1.4 ± 0.2^{a}	1.3 ± 0.3
VSI[7]	6.9 ± 1.7	6.4 ± 2.1	5.8 ± 1.6	5.9 ± 0.8	6.1 ± 1.4	6.5 ± 1.6
Heart weight/body weight ratio (%)	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02
Final whole body composition[8]						
Dry matter	34.6 ± 1.1	34.4 ± 0.4	34.4 ± 1.3	34.2 ± 0.9	34.5 ± 0.7	34.8 ± 0.1
Protein	17.7 ± 0.6	17.3 ± 0.2	17.3 ± 0.4	17.7 ± 0.4	17.6 ± 0.4	17.4 ± 0.4
Lipids	12.6 ± 0.6	13.2 ± 0.4	13.1 ± 1.3	12.8 ± 0.6	12.8 ± 0.6	13.6 ± 0.5
Energy	8.7 ± 0.5	8.9 ± 0.1	8.5 ± 0.1	8.7 ± 0.3	8.7 ± 0.2	9.2 ± 0.4
Ash	4.7 ± 0.6	4.0 ± 0.3	4.1 ± 0.2	4.3 ± 0.6	4.5 ± 0.1	4.5 ± 0.2
Total lipids						
Dorsal muscle	1.3 ± 0.5^{b}	1.3 ± 0.5^{b}	1.5 ± 0.4^{ab}	1.3 ± 0.2^{ab}	1.8 ± 0.6^{a}	1.6 ± 0.4
Ventral muscle	3.7 ± 0.4	2.8 ± 0.1	3.7 ± 0.3	2.3 ± 0.3	3.9 ± 0.6	3.6 ± 0.2
Liver	18.4 ± 3.9^{b}	18.3 ± 0.4^{b}	19.6 ± 4.8^{b}	22.7 ± 4.4^{b}	35.0 ± 1.6^{a}*	25.6 ± 3.7*
Heart	7.3 ± 0.2	7.8 ± 0.6	8.8 ± 0.8	7.9 ± 1.8	9.1 ± 2.0	7.2 ± 1.3
Viscera	75.2 ± 2.8	70.8 ± 6.7	70.9 ± 1.4	75.6 ± 9.9	76.0 ± 4.2	77.0 ± 3.3

Values are presented as mean ± standard deviation (n = 3). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). The presence of an * means significant differences between treatments 100PF and 100PFL ($p < 0.05$). Absence of superscript or absence of * indicates no significant difference between treatments.

[1] K, Condition factor; [2] DGI, Daily growth index; [3] VFI, Voluntary feed intake; [4] FCR, Feed conversion ratio; [5] PER, Protein efficiency ratio; [6] HSI, Hepatosomatic index; [7] VSI, Viscerosomatic index.

[8] Initial body composition: dry matter, 36.8%; protein, 17.1% wet weight (WW); lipids, 15.5% WW; energy, 9.7 kJ g^{-1}WW; ash, 4.6% WW.

After 16 weeks of feeding, all groups more than tripled their initial weight, with final body weights ranging from 62 to 67 g (Table 27). All diets were well accepted by European seabass, resulting in similar VFI and FCR among dietary treatments. Daily growth index, final body weight and final body length were highest in fish fed 50PF, even though these values only differed from those of fish fed 100PF. PF inclusion did not significantly affect the final whole body composition of the fish (Table 27). However, the hepatosomatic index was affected by fish oil replacement, being higher in fish fed 100PF than in those fed other treatments ($p < 0.05$). This increase in the liver size of fish fed 100PF was accompanied by the highest liver total lipids content (Table 27) and higher liver vacuolization degree compared to FO and 25PF fed fish (Table 28; Figure 12). It was also observed that the inclusion of soy lecithin could significantly attenuate liver lipid content in fish fed diets devoid of fish oil (100PFL; Table 27). However, histological analysis showed that glycogen content and vacuolization degree were similar among fish fed 100PF and 100PFL. Liver glycogen content decreased in fish fed 50PF, 75PF and 100PF in relation to those fed the FO diet (Table 28; Figure 13). The total lipid content of the dorsal muscle ranged from 1.3 to 1.8% wet weight (WW), being higher in fish fed 100PF than in those fed FO and 25PF ($p < 0.05$), whereas ventral muscle total lipids did not vary significantly among dietary treatments.

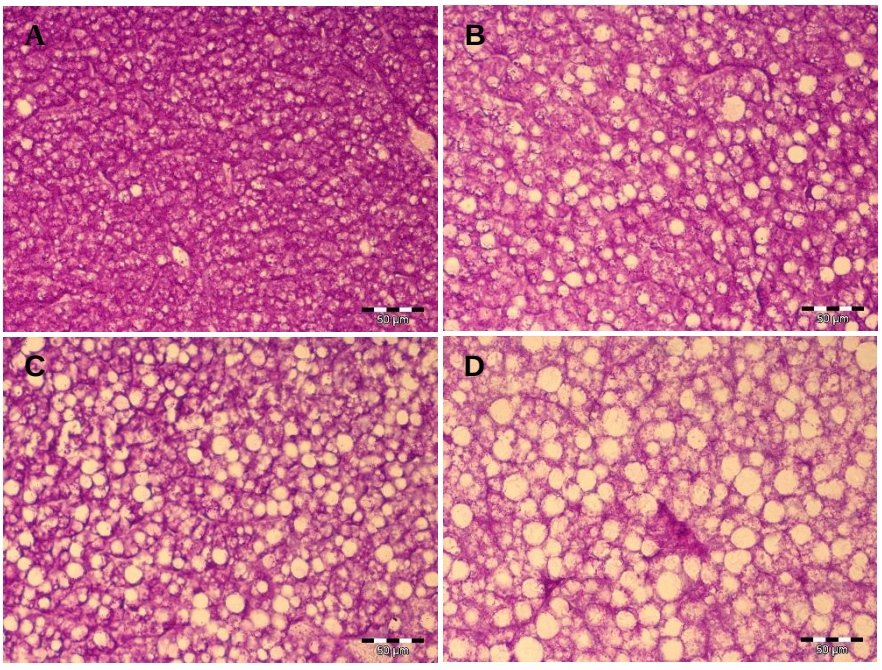

Figure 13. Liver sections of seabass fed FO (A), 25PF (B), 75PF (C) and 100PF (D) (PAS, 40×). Higher hepatocyte cytoplasm pigmentation is observed in fish fed FO-based diet (A), indicating higher amounts of glycogen compared to fish fed the PF-based diet (D).

The fatty acids profile of the different analyzed tissues (dorsal muscle, ventral muscle, liver and heart; Table 29 and Table 30) generally mirrored the dietary fatty acids profile. PF inclusion led to an increase in MUFA and a decrease in PUFA in the dorsal muscle of fish fed 75PF and 100PF, and in the ventral muscle and liver of fish fed 50PF, 75PF and 100PF. MUFA

proportion of heart increased only in fish fed 100PF whereas PUFA decreased in fish fed 75PF and 100PF. Additionally, the *n*-3/*n*-6 ratio decreased significantly with the inclusion of PF in all tissues analyzed. This decrease was significantly attenuated in the heart with the inclusion of soy lecithin in 100PFL. A direct comparison between the most relevant dietary and tissue fatty acids profiles (% total fatty acids) was performed and is portrayed in Figure 14. The linear correlation between fatty acids profile in tissues and in diet evidenced that PUFA are generally preferentially retained by tissues, while SFA are preferentially metabolized (dots above or below the equality line, respectively). The fillet quality indexes estimated in the dorsal and ventral muscle based on their fatty acid profile (Table 29) were affected by the dietary treatments, but this effect was more evident in ventral than in dorsal muscle. The fillet deposition of EPA and DHA (1.6 – 3.5 and 2.1 – 8.1 mg g^{-1} in dorsal and ventral muscle, respectively) and the peroxidation index (102 – 241 and 77 – 188 in dorsal and ventral muscle, respectively), were the parameters most evidently affected by dietary treatments, decreasing with the increase of PF inclusion.

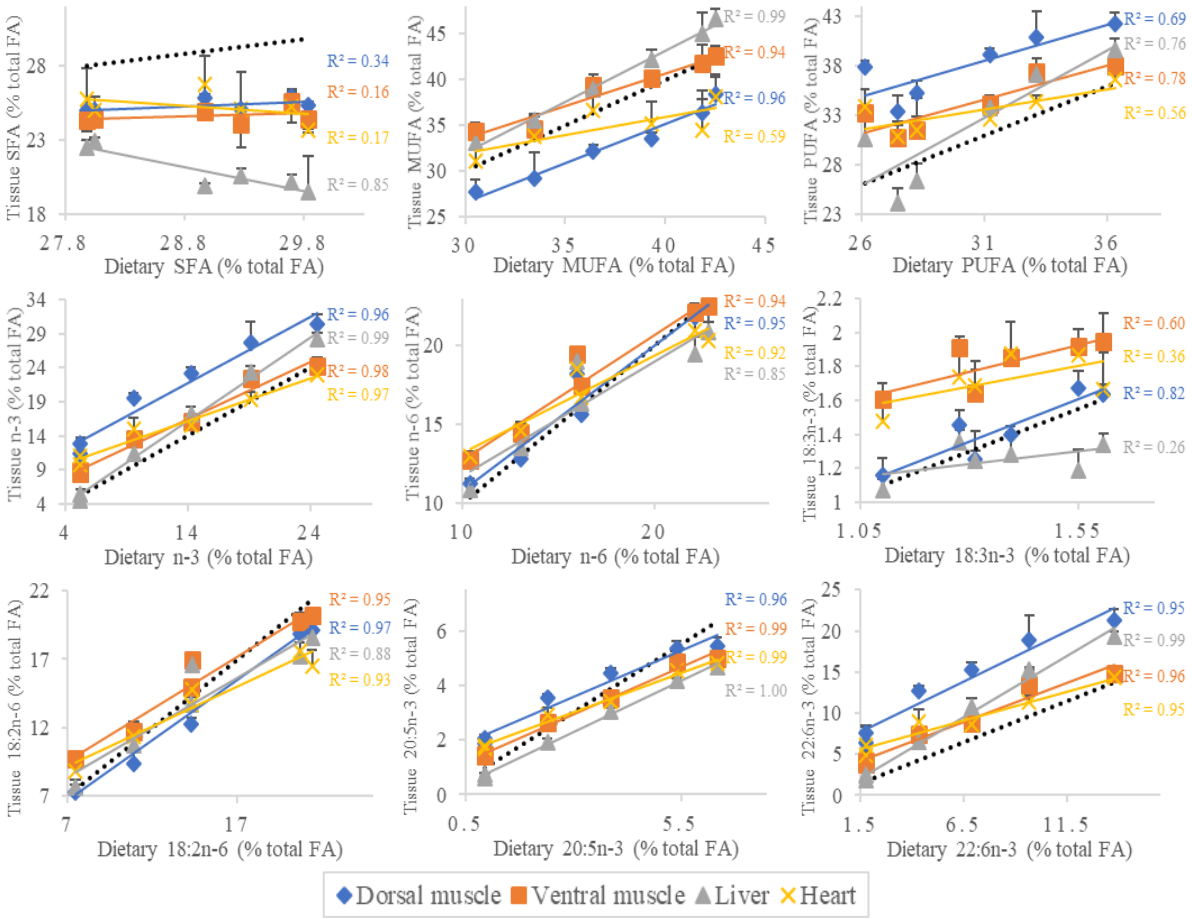

Figure 14. Relationship between dietary and dorsal muscle (diamonds), ventral muscle (squares), liver (triangles) or heart (crosses) fatty acid concentrations for SFA, MUFA, PUFA, *n*-3 fatty acids, *n*-6 fatty acids, α-linolenic (18:3*n*-3), linoleic (18:2*n*-6), eicosapentaenoic (20:5*n*-3) and docosahexaenoic (22:6*n*-3) acids, in European seabass fed the experimental diets for 16 weeks. A line of equality is represented (dashed). Data are represented as mean ± standard deviation. Statistical differences are not represented (see Table 29 and Table 30).

The plasma metabolites at 24 h post-fasting remained unaffected by the dietary treatments (Table 31). However, at 12 h post feeding the levels of triglycerides were significantly higher in fish fed 50PF, 75PF and 100PF compared to those fed FO and 25PF. At 12 h post-feeding the total cholesterol and NEFA were also higher in fish fed 50PF and 100PF than in those fed FO.

The activity of the lipogenesis enzymes assayed is reported as mU g^{-1} hepatic protein in Table 28. G6PD was the enzyme with the highest activity (96 to 156 mU g^{-1}) and was similar among FO and PF diets, but had a significantly lower activity in fish fed 50PF than in those fed 75PF. The activity of this enzyme showed a strong positive correlation with the activity of FAS (r = 0.617). FAS activity was also similar among FO and PF diets, but 50PF fed fish had a lower activity than those fed 100PF. On the other hand, FAS activity was positively correlated with dietary MUFA content (r = 0.517) and with liver lipid content (r = 0.600). The activity of ME was also unaffected by the dietary lipid source and had lowest activity in fish fed 75PF. This enzyme's activity was negatively correlated with dietary SFA content (r = −0.633) and positively correlated with fecal nitrogen losses (r = 0.518), which were highest and lowest in that diet, respectively.

Table 28. Liver vacuolization, glycogen content and specific activity (mU mg^{-1} protein) of selected lipogenic enzymes of European seabass fed the different experimental diets for 16 weeks.

	FO	25PF	50PF	75PF	100PF	100PFL
Vacuolization degree	1.5 ± 0.43	1.6 ± 0.4^{b}	1.8 ± 0.5^{ab}	1.8 ± 0.3^{ab}	2.1 ± 0.2^{a}	1.9 ± 0.3
Glycogen content	2.2 ± 0.3^{a}	1.9 ± 0.3^{ab}	1.8 ± 0.3^{b}	1.8 ± 0.2^{bc}	1.5 ± 0.3^{c}	1.7 ± 0.3
Enzyme activity						
G6PD	103.4 ± 5.6^{ab}	126.1 ± 10.3^{ab}	96.2 ± 2.4^{b}	133.9 ± 12.0^{a}	124.8 ± 6.5^{ab}	158.7 ± 18.6
Malic enzyme	2.3 ± 0.2^{ab}	2.6 ± 0.5^{ab}	2.3 ± 0.4^{ab}	1.4 ± 0.2^{b}	3.0 ± 0.1^{a}	3.2 ± 0.2
FAS	1.2 ± 0.02^{ab}	1.4 ± 0.4^{ab}	0.8 ± 0.1^{b}	1.6 ± 0.2^{ab}	2.2 ± 0.3^{a}	2.0 ± 0.2

Values are presented as mean ± standard deviation or mean ± standard error on enzyme activity (n = 3). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). Absence of superscript or absence of * indicates no significant difference between treatments.

Table 29. Dorsal and ventral muscle fatty acid composition (g 100^{-1} total fatty acid) of the European seabass fed the experimental diets.

	Dorsal muscle							Ventral muscle						
Fatty acids	FO	25PF	50PF	75PF	100PF	100PFL	SEM	FO	25PF	50PF	75PF	100PF	100PFL	SEM
14:0	1.6^{a}	1.5^{a}	1.4^{ab}	1.1^{b}	1.0^{b}	0.9	0.05	2.4^{a}	2.1^{b}	1.9^{c}	1.5^{d}	1.2^{e}	1.2	0.11
16:0	17.2^{d}	17.7^{bc}	17.5^{cd}	18.0^{ab}	18.1^{a}	18.2	0.06	16.5^{c}	17.3^{ab}	16.8^{bc}	17.5^{ab}	17.8^{a}	17.8	0.13
18:0	5.1	5.1	4.9	5.1	5.0	5.0	0.04	4.1	4.6	4.1	4.6	4.6	4.7	0.08
∑ SFA[1]	25.8^{a}	25.9^{a}	25.1^{b}	25.3^{ab}	25.0^{b}	25.0	0.08	24.9^{ab}	25.5^{a}	24.1^{b}	24.4^{ab}	24.4^{ab}	24.4	0.15
16:1*n*-7	2.9	2.9	3.1	3.0	3.5	3.1	0.05	4.0	3.8	4.1	3.7	4.0	3.8	0.05
18:1*n*-9	19.2^{d}	21.0^{cd}	23.9^{bc}	25.8^{b}	30.2^{a}	28.7	0.69	23.4^{c}	24.6^{c}	29.0^{b}	30.8^{b}	33.4^{a}	32.8	0.4
18:1*n*-7	2.2^{a}	2.2^{a}	2.2^{ab}	2.1^{bc}	2.1^{c}	2.0	0.02	2.5^{a}	2.5^{a}	2.5^{a}	2.3^{b}	2.2^{c}	2.2	0.03
20:1*n*-9	1.6	1.5	1.5	1.3	1.4	1.3	0.02	2.3^{a}	2.0^{b}	2.0^{b}	1.8^{bc}	1.7^{c}	1.7	0.05
∑ MUFA[2]	27.7^{c}	29.3^{bc}	32.1^{bc}	33.5^{b}	38.5^{a}	36.4	0.68	34.3^{c}	34.7^{c}	39.4^{b}	40.2^{ab}	42.6^{a}	41.8	0.82
18:2*n*-6	7.3^{e}	9.4^{d}	12.3^{c}	14.8^{b}	18.8^{a}	19.2	0.75	9.7^{e}	11.7^{d}	15.0^{c}	17.0^{b}	19.8^{a}	20.2	0.95
18:3*n*-3	1.2^{c}	1.3^{bc}	1.4^{abc}	1.5^{ab}	1.7^{a}	1.6	0.03	1.6^{c}	1.6^{bc}	1.9^{ab}	1.9^{a}	1.9^{a}	2.0	0.04
20:4*n*-6	2.2^{a}	2.0^{ab}	1.8^{ab}	1.9^{ab}	1.5^{b}	1.7	0.05	1.4^{a}	1.4^{a}	1.0^{ab}	1.1^{ab}	0.9^{b}	1.0	005
20:5*n*-3	5.4^{a}	5.4^{a}	4.5^{b}	3.5^{c}	1.9^{d}	2.1	0.24	5.0^{a}	4.8^{a}	3.5^{b}	2.6^{c}	1.4^{d}	1.5	0.35
22:5*n*-3	1.3^{a}	1.1^{b}	1.0^{bc}	1.0^{c}	0.8^{d}	0.9	0.03	1.3^{a}	1.1^{b}	0.8^{c}	0.7^{c}	0.6^{d}	0.6	0.06
22:6*n*-3	21.3^{a}	18.9^{ab}	15.3^{bc}	12.8^{c}	6.5^{d}	7.6	0.95	14.9^{a}	13.5^{a}	8.8^{b}	7.4^{b}	3.9^{c}	4.2	1.04
∑ PUFA[3]	42.2^{a}	40.9^{ab}	39.1^{ab}	37.9^{b}	33.4^{c}	35.3	0.56	38.0^{a}	37.5^{a}	34.2^{b}	33.3^{b}	30.8^{c}	31.6	0.6
∑ PUFA*n*-6[4]	11.2^{e}	12.8^{d}	15.6^{c}	18.2^{b}	21.8^{a}	22.4	0.71	12.7^{e}	14.4^{d}	17.4^{c}	19.4^{b}	22.1^{a}	22.5	0.89
∑ PUFA*n*-3[5]	30.4^{a}	27.7^{ab}	23.2^{bc}	19.5^{c}	11.4^{d}	12.8	1.22	24.3^{a}	22.4^{a}	16.2^{b}	13.5^{b}	8.5^{c}	8.8	1.50
∑ *n*-3/ ∑ *n*-6	2.7^{a}	2.2^{b}	1.5^{c}	1.1^{c}	0.5^{d}	0.6	0.14	1.9^{a}	1.6^{b}	0.9^{c}	0.7^{c}	0.4^{d}	0.4	0.14
Fillet quality indexes														
EPA + DHA[6]	3.5^{a}	3.1^{a}	2.8^{ab}	2.2^{bc}	1.6^{c}	1.5	0.53	8.1^{a}	5.1^{b}	5.5^{b}	3.2^{c}	2.1^{c}	2.1	0.53
AI[7]	0.34^{a}	0.34^{a}	0.32^{ab}	0.31^{b}	0.31^{b}	0.31	0.004	0.23	0.24	0.23	0.24	0.24	0.24	0.002
TI[8]	0.21^{d}	0.23^{cd}	0.25^{c}	0.28^{b}	0.37^{a}	0.36	0.015	0.21^{e}	0.24^{d}	0.27^{c}	0.31^{b}	0.39^{a}	0.38	0.016
h/H[9]	3.08^{b}	3.08^{b}	3.19^{a}	3.21^{a}	3.21^{a}	3.21	0.018	3.47	3.39	3.57	3.52	3.47	3.49	0.021
PI[10]	240.4^{a}	218.5^{ab}	186.4^{bc}	161.7^{c}	102.2^{d}	113.8	53.40	187.9^{a}	174.2^{a}	128.7^{b}	112.0^{b}	76.7^{c}	79.8	44.60

[1] ∑ SFA, sum of saturated fatty acids, includes 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0;
[2] ∑ MUFA, sum of monounsaturated fatty acids, includes 16:1*n*-9, 14:1*n*-5, 17:1*n*-7, 18:1*n*-9, 18:1*n*-7, 22:1*n*-11;
[3] ∑ PUFA, sum of polyunsaturated fatty acids, includes 16:2*n*-4, 16:3*n*-4, 16:4*n*-1, 18:2*n*-6, 18:2*n*-4, 18:3*n*-3, 18:3*n*-6, 18:4*n*-1, 18:4*n*-3, 20:3*n*-6, 20:3*n*-3, 20:4*n*-3, 20:4*n*-6, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:5*n*-6, 22:6*n*-3;
[4] ∑ PUFA*n*-6, sum of *n*-6 PUFA, includes 18:2*n*-6, 18:3*n*-6, 20:3*n*-6, 20:4*n*-6, 22:5*n*-6;
[5] ∑ PUFA*n*-3, sum of *n*-3 PUFA, includes 18:3*n*-3, 18:4*n*-3, 20:3*n*-3, 20:4*n*-3, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:6*n*-3;
[6] EPA + DHA = eicosapentaenoic acid (20:5*n*-3) + docosahexaenoic acid (22:6*n*-3), expressed in mg g^{-1} muscle wet weight;
[7] AI, Atherogenicity index; [8] TI, Thrombogenicity index; [9] h/H, Hypocolesterolemic and hypercolesterolemic fatty acids ratio; [10] PI, Peroxidation index;
Values are presented as mean ± standard error of the mean (n = 3). For each tissue presented, values in the same row without a common superscript letter differ significantly ($p < 0.05$). The presence of an * means significant differences between treatments 100PF and 100PFL ($p < 0.05$). Absence of superscript or absence of * indicates no significant difference between treatments.

Table 30. Liver and heart fatty acid composition (g/100 g total fatty acid) of the European seabass fed the experimental diets.

	Liver							Heart						
	FO	25PF	50PF	75PF	100PF	100PFL	SEM	FO	25PF	50PF	75PF	100PF	100PFL	SEM
14:0	1.7^{a}	1.5^{b}	1.4^{c}	1.2^{d}	1.2^{d}	1.0	0.06	2.1^{a}	2.0^{a}	1.7^{b}	1.2^{c}	1.1^{c}	1.2	0.10
16:0	13.2^{d}	13.7^{cd}	14.3^{bc}	14.9^{b}	16.3^{a}	16.1	0.29	15.9^{b}	16.1^{ab}	16.2^{ab}	16.0^{ab}	16.8^{a}	16.3	0.10
18:0	3.4	3.6	3.7	3.7	4.6	4.5	0.13	5.8	5.1	5.5	5.2	5.8	6.5	0.5
∑ SFA[1]	19.9^{b}	20.1^{ab}	20.5^{ab}	19.5^{b}	22.9^{a}	22.5	0.38	26.8	25.2	25.1	23.6	25.0	25.7	0.40
16:1*n*-7	4.1^{bc}	4.0^{c}	4.3^{ab}	4.4^{a}	4.4^{a}	4.3	0.04	3.3	3.5	3.4	3.1	3.3	3.0	0.07
18:1*n*-9	23.7^{d}	26.4^{d}	29.8^{c}	33.3^{b}	37.9^{a}	36.5	1.28	21.3^{c}	24.1^{bc}	27.4^{ab}	26.6^{ab}	29.7^{a}	26.7	0.78
18:1*n*-7	2.8^{a}	2.8^{a}	2.7^{b}	2.5^{c}	2.4^{d}	2.4	0.04	2.4	2.4	2.4	2.3	2.2	2.1	0.04
20:1*n*-9	1.5^{a}	1.4^{ab}	1.3^{bc}	1.2^{c}	1.2^{c}	1.2	0.03	1.9	1.9	1.9	1.6	1.6	1.4	0.05
∑ MUFA[2]	33.1^{d}	35.6^{d}	39.0^{c}	42.2^{b}	46.7^{a}	45.1	1.21	31.1^{b}	33.8^{ab}	36.6^{ab}	35.2^{ab}	38.1^{a}	34.5	0.74
18:2*n*-6	7.7^{d}	10.8^{c}	13.7^{b}	16.6^{a}	17.2^{a}	18.6	0.94	8.8^{d}	11.4^{c}	13.7^{b}	14.8^{b}	17.6^{a}	16.5	0.74
18:3*n*-3	1.1^{b}	1.2^{ab}	1.3^{ab}	1.4^{a}	0.19^{ab}	1.3	0.03	1.5	1.7	1.9	1.7	1.9	1.7	0.05
20:4*n*-6	1.5^{a}	1.2^{ab}	1.1^{bc}	0.9^{c}	0.6^{d}	0.7	0.08	2.5	2.0	1.8	2.5	2.2	2.6	0.12
20:5*n*-3	4.7^{a}	4.2^{b}	3.1^{c}	1.9^{d}	0.6^{e}	0.7	0.38	4.8^{a}	4.5^{a}	3.4^{b}	2.9^{b}	1.7^{c}	1.8	0.30
22:5*n*-3	1.6^{a}	1.3^{b}	1.0^{c}	0.7^{d}	0.3^{e}	0.4	0.11	1.2^{a}	1.0^{b}	0.9^{b}	0.9^{b}	0.8^{b}	0.9	0.04
22:6*n*-3	19.4^{a}	15.2^{b}	10.7^{c}	6.6^{d}	1.9^{e}	2.4	1.57	14.3^{a}	11.4^{b}	8.7^{c}	8.9^{c}	4.9^{d}	6.1	0.80
∑ PUFA[3]	39.7^{a}	37.2^{ab}	33.8^{bc}	30.6^{c}	24.1^{d}	26.4	1.37	36.6^{a}	34.4^{ab}	32.6^{abc}	33.9^{bc}	30.9^{c}	31.5	0.58
∑ PUFA*n*-6[4]	10.8^{d}	13.5^{c}	16.3^{b}	19.0^{a}	19.4^{a}	20.8	0.87	13.0^{d}	14.6^{d}	16.7^{c}	18.5^{b}	20.9^{a}	20.3	0.72
∑ PUFA*n*-3[5]	28.2^{a}	23.2^{b}	17.1^{c}	11.4^{d}	4.5^{e}	5.4	2.14	23.0^{a}	19.3^{b}	15.5^{c}	15.1^{c}	9.8^{d}	11.0	1.14
∑ *n*-3/ ∑ *n*-6	2.6^{a}	1.7^{b}	1.1^{c}	0.6^{d}	0.2^{e}	0.3	0.21	1.8^{a}	1.3^{b}	0.9^{c}	0.8^{c}	0.47^{d}*	0.54*	0.11

[1] ∑ SFA, sum of saturated fatty acids, includes 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0;

[2] ∑ MUFA, sum of monounsaturated fatty acids, includes 16:1*n*-9, 14:1*n*-5, 17:1*n*-7, 18:1*n*-9, 18:1*n*-7, 22:1*n*-11;

[3] ∑ PUFA, sum of polyunsaturated fatty acids, includes 16:2*n*-4, 16:3*n*-4, 16:4*n*-1, 18:2*n*-6, 18:2*n*-4, 18:3*n*-3, 18:3*n*-6, 18:4*n*-1, 18:4*n*-3, 20:3*n*-6, 20:3*n*-3, 20:4*n*-3, 20:4*n*-6, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:5*n*-6, 22:6*n*-3;

[4] ∑ PUFA*n*-6, sum of *n*-6 PUFA, includes 18:2*n*-6, 18:3*n*-6, 20:3*n*-6, 20:4*n*-6, 22:5*n*-6;

[5] ∑ PUFA*n*-3, sum of *n*-3 PUFA, includes 18:3*n*-3, 18:4*n*-3, 20:3*n*-3, 20:4*n*-3, 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:6*n*-3;

Values are presented as mean ± standard error of the mean (n = 3). For each tissue presented, values in the same row without a common superscript letter differ significantly ($p < 0.05$). The presence of an * means significant differences between treatments 100PF and 100PFL ($p < 0.05$). Absence of superscript or absence of * indicates no significant difference between treatments.

Table 31. Plasma glucose, total cholesterol, triglycerides (mg dL^{-1}) and non-esterified fatty acids (mmol L^{-1}) up to 24h after the last meal.

Diet	Time (h)	Glucose	Total cholesterol	Triglycerides	Non-esterified fatty acids
FO	2	87.4 ± 9.1^{B}	155.9 ± 4.3^{cd}	42.0 ± 2.9^{cde}	0.46 ± 0.05^{cde}
	6	112.4 ± 11.1^{AB}	153.4 ± 7.2^{cd}	53.3 ± 2.5^{c}	0.73 ± 0.07^{cde}
	12	120.7 ± 12.8^{B}	158.0 ± 5.3^{cd}	47.3 ± 2.7^{cde}	0.78 ± 0.10^{bcde}
	24	109.7 ± 13.1^{A}	170.4 ± 7.7^{bcd}	35.1 ± 5.9^{defg}	0.25 ± 0.02^{de}
25PF	2	100.0 ± 6.2^{B}	158.9 ± 11.3^{cd}	47.4 ± 7.4^{cdef}	0.58 ± 0.10^{cde}
	6	104.1 ± 9.9^{AB}	153.5 ± 11.7^{cd}	53.4 ± 1.8^{bcd}	0.73 ± 0.09^{bcde}
	12	109.9 ± 13.3^{B}	174.2 ± 3.1^{bcd}	48.3 ± 3.2^{cd}	0.78 ± 0.10^{bcde}
	24	105.5 ± 5.1^{A}	178.8 ± 6.7^{bcd}	23.7 ± 1.1^{efg}	0.25 ± 0.02^{de}
50PF	2	114.6 ± 5.8^{B}	166.4 ± 9.1^{bcd}	45.5 ± 6.5^{abc}	0.99 ± 0.09^{bcd}
	6	128.1 ± 12.4^{AB}	160.4 ± 8.9^{bcd}	46.4 ± 3.5^{cd}	1.13 ± 0.25^{bcde}
	12	104.0 ± 5.0^{B}	256.6 ± 8.6^{a}	77.5 ± 4.6^{a}	1.95 ± 0.10^{a}
	24	114.7 ± 10.2^{A}	167.5 ± 2.4^{bcd}	16.0 ± 2.8^{g}	0.25 ± 0.02^{de}
75PF	2	92.7 ± 10.2^{B}	152.8 ± 8.0^{cd}	52.7 ± 3.5^{cde}	0.79 ± 0.08^{cde}
	6	110.5 ± 4.6^{AB}	174.6 ± 8.2^{bcd}	50.1 ± 5.5^{bc}	0.95 ± 0.20^{bcd}
	12	85.5 ± 5.6^{B}	186.2 ± 8.5^{bc}	72.8 ± 2.5^{ab}	1.04 ± 0.14^{bc}
	24	128.3 ± 6.4^{A}	166.1 ± 5.0^{bcd}	21.9 ± 3.9^{fg}	0.28 ± 0.04^{e}
100PF	2	77.3 ± 6.7^{B}	141.9 ± 7.9^{d}	45.4 ± 2.2^{cde}	0.61 ± 0.05^{cde}
	6	107.2 ± 11.2^{AB}	177.1 ± 9.7^{bcd}	50.3 ± 4.1^{c}	0.70 ± 0.09^{cde}
	12	104.3 ± 9.2^{B}	198.9 ± 11.9^{b}	67.9 ± 5.2^{a}	1.40 ± 0.20^{ab}
	24	133.8 ± 10.8^{A}	174.4 ± 7.0^{bcd}	34.7 ± 4.1^{defg}	0.45 ± 0.05^{cde}
Two-way ANOVA p-value	Diet	0.379	0.001	0.008	0.001
	Time	0.005	0.000	0.000	0.000
	Diet x Time	0.167	0.000	0.000	0.001
100PF	2	77.3 ± 6.7^{C}	141.9 ± 7.9^{B}	45.4 ± 2.2^{de}	0.61 ± 0.05^{B}
	6	107.2 ± 11.2^{AB}	177.1 ± 9.7^{B}	50.3 ± 4.1^{cd}	0.70 ± 0.09^{BC}
	12	104.3 ± 9.2^{B}	198.9 ± 11.9^{A}	67.9 ± 5.2^{a}	1.40 ± 0.20^{A}
	24	133.8 ± 10.8^{A}	174.4 ± 7.0^{AB}	34.7 ± 4.1^{ef}	0.45 ± 0.05^{C}
100PFL	2	70.1 ± 5.4^{C}	161.40 ± 10.4^{B}	68.8 ± 2.0^{ab}	1.02 ± 0.07^{B}
	6	108.9 ± 9.1^{AB}	158.2 ± 9.7^{B}	57.2 ± 3.1^{bc}	0.78 ± 0.08^{BC}
	12	106.6 ± 7.1^{B}	207.2 ± 14.8^{A}	73.5 ± 4.0^{ab}	1.40 ± 0.21^{A}
	24	132.9 ± 2.6^{A}	196.0 ± 5.3^{AB}	21.2 ± 2.6^{f}	0.34 ± 0.06^{C}
Two-way ANOVA p-value	Diet	0.879	0.272	0.027	0.461
	Time	0.000	0.001	0.000	0.000
	Diet x Time	0.806	0.263	0.000	0.398

Values are presented as mean ± standard error (n = 6). Different upper-case letters indicate differences among sampling time. Different lower-case letters indicate interactions between the two factors (diet *vs* time).

Discussion

All experimental diets were well digested by seabass, and the replacement of fish oil by PF had no effect on the ADC of nutrients. Similarly, in Atlantic halibut (*H. hippoglossus*) no differences were reported in the ADC of protein and lipids between a fish oil-based diet and a PF-based diet, but in this species MUFA ADC has also decreased in the PF diet (Martins et al., 2009). Although in the present study some differences were registered in the faecal nutrient losses among experimental diets (higher faecal N and lipid losses in fish fed 100PF diet compared to fish fed 75PF), nutrients gain and retention efficiencies remained similar in all groups. Moreover, the observed results are within the range of values previously reported for this species fed fishmeal-based diets (Campos et al., 2017; Peres & Oliva-Teles, 1999; Peres & Oliva-Teles, 2005; Santos et al., 2010; Valente et al., 2006). These data indicate that fish oil replacement by PF did not impair the dietary utilization of N, lipids and energy.

Fish oil was totally replaced by PF without impairing growth of Japanese seabass (*Lateolabrax japonicus*), yellowtail kingfish (*Seriola lalandi*), barramundi (*Lates calcarifer*), vundu (*Heterobranchus longifilis*), and largemouth bass (*Micropterus salmoides*; Ahmad et al. (2013); Babalola et al. (2009); Bowyer et al. (2012); Xue et al. (2006); Yun et al. (2013)). Likewise, in the present study, final body weight and growth performance of seabass were not significantly affected by the replacement of fish oil by PF even when fish oil was totally replaced (100PF). Contrarily, the total replacement of fish oil by a blend of PF and mammal fat impaired growth in European seabass (Monteiro et al., 2018), suggesting that PF is a better fat source for this species.

The final whole body composition of fish remained unaffected among experimental diets, which is consistent with previous reports on the use of PF as partial or total fish oil replacement in yellowtail kingfish, Japanese seabass and largemouth bass (Bowyer et al., 2012; Xue et al., 2006; Yun et al., 2013) and with the use of a blend of PF and mammal fat in European seabass (Monteiro et al., 2018). In those studies, the hepatosomatic index and liver lipid content increased when 50% of the fish oil was replaced with PF in Japanese seabass (Xue et al., 2006) and when fish oil was completely replaced by a blend of PF and mammal fat in European seabass (Monteiro et al., 2018), while largemouth bass fed PF-based diets showed no alteration of hepatosomatic index and liver lipid content (Yun et al., 2013). However, the present study showed that only when fish oil was completely replaced by PF lipid deposition increased in liver (compared to FO fed fish) and in dorsal muscle (compared to FO and 25PF), but not in ventral muscle, viscera and heart of European seabass. Fish fed 100PF also had higher vacuolization degree and lower glycogen content in the hepatocytes, which is consistent with its higher lipid deposition and increased hepatosomatic index, confirming previous results

in the same species where a blend of PF and mammal fat was used to replace 100% fish oil (Monteiro et al., 2018). This difference among studies may be due not only to different dietary lipid content (in largemouth bass dietary lipid was about 9% while in the present study diets had about 19% dietary lipids) but it can also be due to differential lipid deposition areas among species. In Japanese seabass, for instance, it has been shown that the preferential lipid deposition site is in the adipose tissue and liver (Ren et al., 2017), which can explain the effects observed at hepatic level at 50% fish oil replacement by PF observed by Xue et al. (2006). In the present study, the accumulation of lipids in the liver may have been caused by insufficient amounts of phospholipids required for the lipoprotein synthesis, which can explain the attenuation of this deposition when feed was supplemented with soy lecithin. This has been previously suggested by Olsen et al. (1999) in Arctic char (*Salvelinus alpinus*). On the other hand, the lipid accumulation in the dorsal muscle may have been due to the high dietary levels of 18:2*n*-6 and 18:3*n*-3 in 100PF, which are known to have a direct absorption and esterification in the muscle and also a high mobilization from liver to muscle (Henderson, 1996). No lipid accumulation was observed on ventral muscle, which may be due to its higher lipid content than dorsal muscle.

The fatty acids profiles of the European seabass tissues were affected by the inclusion of PF and generally reflected the dietary fatty acids profiles, as in most fish species (Trushenski & Lochmann, 2009; Turchini et al., 2009), leading to increased MUFA levels, reduced PUFA proportions and a decreased *n*-3/*n*-6 ratio in all tissues except the heart, which maintained relatively stable fatty acid profiles with the PF inclusion. There are no literature reports on the ideal dietary n-3/*n*-6 ratio for seabass juveniles, but in larvae Sargent et al. (1999) reported that dietary 20:5*n*-3/20:4*n*-6 (EPA/ARA) ratio should not be below 1. In the present study all diets had a 20:5*n*-3/20:4*n*-6 ratio over 1.5. Although ARA is essential to regulate the stress response in seabass (Montero et al., 2015), high levels of this fatty acid compete with EPA and decrease its deposition in the liver and brain, ultimately inducing neural development abnormalities (Bell et al., 1995). On the other hand, a low dietary *n*-3/*n*-6 ratio (0.4) resulted in increased inflammatory responses in Atlantic salmon (Salmo salar) when compared to fish fed a higher (1.4) dietary *n*-3/*n*-6 ratio (Holen et al., 2018). Although in the present study the effects of *n*-3/*n*-6 ratios on the immunological status were not evaluated, they could also possibly have impacted the sea bass inflammatory responses. In all tissues, the reduction of total PUFA with increasing dietary PF was the result of a strong reduction in dietary *n*-3 PUFA levels, in spite of increased *n*-6 PUFA levels in such diets (in the form of 18:2*n*-6). This resulted in a dramatic reduction in the *n*-3/*n*-6 ratio paralleled by the fish oil replacement level. Therefore, even though fish tissues still had overall high total PUFA levels, the *n*-3/*n*-6 ratio and consequently the nutritional value of the muscle associated with *n*-3 PUFA consumption were completely

altered. These results are consistent with previous reports on the use of PF in European seabass, barramundi, largemouth bass, Japanese seabass, yellowtail kingfish and Atlantic salmon (Ahmad et al., 2013; Bowyer et al., 2012; Monteiro et al., 2018; Xue et al., 2006; Yun et al., 2013) that also reported a decreased *n*-3/*n*-6 ratio, decreased total PUFA levels and increased total MUFA levels in either muscle or liver. On the other hand, decreased levels of SFA in the diet with increasing PF, resulted in decreased SFA levels in the muscle, but increased levels in the liver. This differential impact of dietary SFA on tissue composition has been previously reported and seems to vary among species (Turchini et al., 2009). In the liver, this might have been a result of the increased FAS activity in fish fed high levels of PF, which resulted in a higher *de novo* synthesis of 16:0 and ultimately higher hepatic SFA levels. On the other hand, the increased FAS activity in fish fed high PF levels did not result in higher 18:0 tissue levels, which is also a product of that enzyme activity (Tocher, 2003), but a significant increase in 18:1*n*-9 was observed in all analysed tissues. SFA can be converted to MUFA by fish, more specifically, 16:0 and 18:0 can be desaturated into 16:1*n*-7 and 18:1*n*-9, respectively, while 20:0 can be elongated into 20:1*n*-9 (NRC, 2011). The increased levels of 18:1*n*-9 observed with increasing dietary PF suggest an endogenous origin of this fatty acid, as levels were always above those provided by diets. Contrarily, the decreased muscle SFA levels did not reflect neither the dietary SFA levels nor an increase in FAS activity. A preferential use of SFA for energy production in fish (Henderson & Sargent, 1985) might explain the reported decrease of SFA in the dorsal muscle.

The levels of *n*-3 fatty acids in the muscle were strongly affected by the fish oil replacement; however, EPA and DHA were selectively retained in the muscle, which is evidenced by the higher levels of these fatty acids in the muscle than in the diets, particularly in the dorsal muscle, and more evidently for DHA than for EPA. This was also reported in European seabass when a blend of PF and mammal fat was used to replace fish oil (Monteiro et al., 2018). This might be a result of a higher peroxisomal β-oxidation of EPA in detriment of DHA, due to the complex catabolism of this fatty acid (Bell et al., 2001; Madsen et al., 1998). Also, Henderson and Sargent (1985) reported a preferential use of 16:0 and 18:1*n*-9 for energy production on the mitochondrial system of fish in detriment of other fatty acids such as PUFA. Since these two fatty acids increased concomitantly with the increased PF inclusion, this may explain the preferential retention of other fatty acids such as *n*-3 PUFA. Furthermore, the recommended levels of EPA and DHA to decrease the risk of cardiovascular diseases in humans (0.25 – 0.5 g per 100 g portion of fish; EFSA Panel on Dietetic Products and Allergies (2010)) were still achieved in dorsal muscle with replacements of fish oil by PF up to 50% and up to 75% in ventral muscle.

Even though in the present study fish did not reach commercial size, for a better perception of the PF impact on the fillet quality, several indexes were estimated for dorsal and ventral muscle, regarding the putative impacts on the consumer's coronary heart health (index of atherogenicity, index of thrombogenecity and hypocholesterolemic and hypercholesterolemic fatty acids ratio; Ulbricht and Southgate (1991)), and on the stability of the fish fillet (peroxidation index; Hulbert et al. (2007)). When PF replaced 75 or 100% of the fish oil, the seabass dorsal muscle showed lower atherogenicity index, indicating a lower risk of lipid deposition in blood vessels (Ulbricht & Southgate, 1991). However, at these replacement levels, dorsal muscle EPA and DHA were below recommended levels for human consumption. In European seabass fed diets with a blend of PF and mammal fat, there were no alterations on the atherogenicity index (Monteiro et al., 2018), indicating that mammal fat does not contribute to decrease this index. Furthermore, the hypocholesterolemic/hypercholesterolemic fatty acids ratio increased in the dorsal muscle of fish fed diets with 50, 75 and 100% fish oil replacement, indicating that the consumption of fillets from these fish may reduce the hypercholesterolemia risk in humans. Additionally, since MUFA are more resistant than PUFA to peroxidation (Hulbert et al., 2007), the overall decrease in PUFA and increase in MUFA led to strong reductions in the peroxidation index of both dorsal and ventral muscle when fish oil was replaced above 50%. This also suggests that fillets from European seabass fed diets with 50% or more fish oil replaced by PF may have a longer shelf life than those fed fish oil-based diets, similarly to what was reported in barramundi fed PF-based diets (Ahmad et al., 2013). Considering all indicators, we may hypothesize that a diet with 50% PF might be able to provide the consumer a seabass dorsal muscle with the recommended levels of EPA and DHA and reduce the risk of lipid deposition in blood vessels and hypercholesterolemia, thus improving consumer's coronary heart health.

Campos et al. (2016) have recently reported similar digestibility of total lipids, total MUFA and total SFA in European seabass fed either PF or fish oil-based diets, and an overall high individual fatty acids digestibility was reported in PF (90%). Furthermore, the high triglycerides level obtained 2 hours after feeding in the present study suggest that these lipids are also easily absorbed by seabass. Therefore, a direct comparison between dietary and tissue fatty acids profile (% total fatty acids) might provide useful information regarding their metabolic destination. Results show that even though most tissues have a linear correlation with dietary fatty acids levels, the heart is less affected by dietary profile than other tissues. This is in accordance with previous results in Atlantic halibut that reported a weaker correlation between dietary and heart fatty acids (Martins et al., 2011). Not only does the heart have low lipid levels, but lipids present in the heart tissues are mainly polar lipids (Kwetegyeka et al., 2011) and it has been shown that the polar lipids fraction is less susceptible to reflect the

dietary fatty acids profile than the storage fraction (neutral lipids) of tissue lipids (Brodtkorb et al., 1997). Moreover, the equality line in each graph indicates if a particular fatty acid is preferentially retained or hydrolyzed (dots above or below that line, respectively) in each tissue. SFA and MUFA have been previously identified as preferential substrates for β-oxidation in fish, over PUFA, which are preferentially deposited in fish tissues (Stubhaug et al., 2007). In the present study, dietary SFA and MUFA were preferentially metabolized in most tissues as their dietary level increased, indicating a use of dietary surplus for energy production, as previously suggested by other authors (Bell et al., 2003a; Stubhaug et al., 2007). However, in the liver MUFA were mainly deposited irrespectively of the diet. Dietary PUFA were preferentially retained and deposited in most tissues, and not selectively used as energy sources. Moreover, *n*-3 fatty acids were preferentially deposited in dorsal and ventral muscle tissues irrespectively of their dietary level, while increasing levels of *n*-6 fatty acids in diets led to a preferential hydrolysis of these fatty acids. Studies with Atlantic salmon have shown that 18:3*n*-3 and 18:2*n*-6 were readily oxidized when at high dietary concentrations (Bell et al., 2003a; 2003b; Stubhaug et al., 2007; Torstensen et al., 2004). In the present study, 18:2*n*-6 was readily oxidized in seabass but not 18:3*n*-3, which was only preferentially hydrolyzed in the liver. This result might be explained by the lower dietary levels of 18:3*n*-3 presently tested compared to the above-mentioned literature (2 *vs* 8% total fatty acids). Moreover, results obtained in the present study indicate that when dietary EPA levels are low, this fatty acid is preferentially deposited in dorsal and ventral muscle and in the heart, while at higher dietary levels EPA is metabolized. DHA, on the other hand, is preferentially deposited in all tissues, irrespectively of its dietary content. According to Stubhaug et al. (2007), both EPA and DHA are highly β-oxidized in Atlantic salmon when in dietary surplus, but preferentially retained when dietary levels are low. Since European seabass is a marine fish species unable to synthetize DHA, it requires higher dietary DHA levels than Atlantic salmon, which could explain its selective retention.

All plasma metabolites assessed in this trial (glucose, cholesterol, triglycerides and NEFA) were unaffected by the dietary treatments at 24 h post-feeding, suggesting that the dietary lipid source did not affect the basal plasmatic levels of these metabolites in European seabass. Similar results were previously reported in this species when fed alternative lipid sources, including a blend of PF and mammal fat (Castro et al., 2015; Monteiro et al., 2018). Although decreased plasma cholesterol levels were reported in *Heterobranchus longifilis* fed PF-based diets (Babalola et al., 2009), this hypocholesterolemic effect was not herein observed. European seabass fed 50PF had significantly higher plasma cholesterol levels at 12 h post-feeding than fish fed FO, whereas the muscle presented increased hypocholesterolemic potential for humans. In this study, the plasmatic cholesterol levels were

positively correlated (r = 0.570) with dietary 18:3*n*-3, which is a fatty acid associated with hypocholesterolemia in mammals (Morise et al., 2004). This suggests that 18:3*n*-3 may have contradictory cholesterolemic effects on mammals and on fish. Similar results have been shown in blackspot seabream (*Pagellus bogaraveo*), a species that showed lower plasma cholesterol levels when fed diets with lower amounts of 18:3*n*-3 (Figueiredo-Silva et al., 2010a). After 12 fasting hours, the levels of triglycerides were significantly higher in fish fed 50PF, 75PF and 100PF compared to FO diet. Since the dietary absorption of triglycerides was similar (indicated by the similar lipid ADC's among experimental diets) these results suggest that endogenous production of triglycerides was increased when fish oil was replaced by 50% or more PF. The levels of NEFA were only significantly different among experimental diets at 12 h post-feeding, when plasmatic levels were higher in fish fed 50PF, 100PF and 100PFL than in fish fed FO, suggesting a higher lipogenic capacity in fish fed those diets.

G6PD was the enzyme involved in lipogenesis with highest activity and was unaffected by the dietary lipid source. G6PD activity was almost 50 times higher than that of ME, suggesting that the pentose-phosphate pathway is the main NADPH source for lipogenesis. The lower activity of ME compared to G6PD had been previously reported in European seabass (Dias et al., 1998; Monteiro et al., 2018) and in salmonids (Hung & Storebakken, 1994; Lin et al., 1977a; Lin et al., 1977b; Walzem et al., 1991). FAS activity was positively correlated with dietary MUFA content (r = 0.517) and with liver lipid content (r = 0.600). The effect of lipid source on lipid metabolism and, consequently, on the liver lipid content has been previously suggested in a study with the use of rendered animal fats in diets for rainbow trout, where histological liver alterations were observed (Caballero et al., 2002). While Monteiro et al. (2018) reported a decrease in ME activity when fish oil was completely replaced by 50% PF + 50% mammal fat, in the present study this enzyme's activity was not affected by the dietary treatment, suggesting that the reduction in the previous study was induced by the mammal fat. The activities of ME and FAS were positively correlated (r = 0.617) and not affected by the dietary lipid source, but FAS activity in fish fed 50PF was lower than in those fed 100PF. Figueiredo-Silva et al. (2010b) showed that, in blackspot seabream (*Pagelus bogaraveo*), the FAS activity was positively correlated with VFI. In the present study, even though that correlation was not significant, fish fed 50PF had the lowest VFI and FAS activity. Furthermore, evidences showed that acetyl-CoA resultant from lipid β-oxidation is primarily directed into energy production and, since it is the primary substrate for lipogenesis, the lack of this molecule has an inhibitory effect on lipogenesis (Nelson et al., 2008). Since fish fed 50PF had simultaneously the lowest VFI, as well as a lower digestible nutrient intake than most diets, and the highest final body weight, this suggests that the acetyl-CoA produced by these

fish may have been preferentially directed into growth, resulting in a down-regulated FAS activity.

The effects of including an emulsifier (soy lecithin) in the PF-based diet were evident in the attenuation of liver lipid deposition (26% WW in 100PFL *vs* 35% in 100PF). The inclusion of soy lecithin also significantly attenuated the decrease in the heart *n*-3/*n*-6 HUFA ratio while inducing a better digestibility of triglycerides, as indicated by a higher plasma triglycerides level in fish fed 100PFL, 2 h after feeding, than in fish fed 100PF. Soy lecithin was added as an emulsifier to aid in the absorption of PF, since this fat has a high melting point (about 28 ºC) that could result in digestibility issues (Bureau et al., 2002). This study was conducted at a relatively warm water temperature (21 ± 1 ºC), which might have helped overcome potential PF digestibility issues. Since European seabass may be reared in a wide temperature range (5 – 28 ºC; FAO (2017)), soy lecithin could be a valuable additive to PF diets when used in lower water temperatures.

The present study showed that the total replacement of fish oil by PF is possible without impairing European seabass juveniles' growth performance and nutrient gain. However, even though supplemental fish oil has been completely replaced, it is important to mention that all essential fatty acids are still provided by the fishmeal included in such diets. A diet with 50% PF was not only able to provide the recommended levels of EPA and DHA for human consumption, but to reduce the risk of lipid deposition and hypercholesterolemia, potentially improving the consumer's coronary heart health. However, total replacement of fish oil by PF resulted in increased lipid accumulation in liver and reduced levels of muscle omega-3, but soy lecithin could be a valuable additive to such PF diets when used in lower water temperatures. Since PF was obtained by rendering locally produced by-products, its use could diminish the carbon footprint of the aquafeed sector contributing to a circular economy concept. The long-term effects of using PF as fish oil replacement still warrant further evaluation. Moreover, such low *n*-3 dietary levels should also be tested in pilot trials under variable temperature and salinity conditions, to fully understand their impact on seabass growth performance and health status

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Chapter VI

A blend of land animal fats can replace up to 75% fish oil without affecting growth and nutrient utilization of European seabass

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A blend of land animal fats can replace up to 75% fish oil without affecting growth and nutrient utilization of European seabass

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Abstract

Animal fats of terrestrial origin are often by-products from agro-food industries that could be valuable sustainable sources of lipids for aquafeeds. Four diets were hence tested in European seabass (*Dicentrarchus labrax*) juveniles (initial body weight, 20 g): a control diet with fish oil (FO) and three diets where a blend of poultry and mammal fats (50:50) replaced 50, 75 and 100% of the supplemental fish oil (50PFMF, 75PFMF and 100PFMF). All diets were isoproteic (51% dry matter, DM) and isoenergetic (23 kJ g^{-1} DM). After 114 days of feeding the experimental diets, the apparent digestibility coefficients (ADCs) of nutrients were determined and fish growth performance evaluated. Postprandial plasma metabolites and muscle fatty acid profile were determined. Liver was also sampled for histologic evaluation and determination of lipogenic enzymatic activity. Protein and energy ADCs were not affected by the dietary treatments, but lipid ADC was lowest in the diet devoid of fish oil (100PFMF). Replacement of fish oil by a blend of land animal fats did not affect daily growth index, feed conversion ratio or nutrient utilization. Whole body composition remained unaffected by dietary treatments, but there was a significant increase in the hepatosomatic index of fish fed with 100PFMF. Total replacement of fish oil by PFMF resulted in increased hepatic vacuolation, apparent steatosis and compromised glycogen deposition. Malic enzyme activity was lowest in fish fed 100PFMF. A significant reduction of muscle eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) content was observed with decreasing levels of fish oil. Results indicate that juvenile seabass can effectively use diets with high levels of land animal fats as alternative lipid source, up to 75% fish oil replacement, without impairing nutrient intake, growth performance and nutrient utilization. Despite alteration in the muscle fatty acid profile, European seabass fed up to 75% PFMF still provides the recommended daily intake levels of EPA + DHA (0.3 – 0.5 g per 100 g of muscle) for human consumption and presents low atherogenecity and thrombogenegity indexes (< 1). Thus, European seabass fed with up to 75% land animal fats can still be considered a good nutritional value end-product for human consumption.

Introduction

European seabass (*Dicentrarchus labrax*) is one of the most commercially valuable species in the Mediterranean countries and is highly appreciated by consumers (EUMOFA, 2016). In 2015, over 150 thousand tonnes were produced, mainly in Greece, Turkey and Spain (FEAP, 2016). It is well known that the progressive growth in global finfish aquaculture exacerbated the exploitation of marine resources for fishmeal and fish oil to incorporate in aquafeeds (Tacon and Metian, 2008). In fact, diets for carnivorous species of higher trophic levels, including European seabass, are still heavily dependent on fish oil as a source of energy and essential fatty acids (Tacon et al., 2011). Nevertheless, the current level of marine fish oil used worldwide by the expanding aquaculture industry has led to major concerns, not only due to the dramatic price increases of fish oil, but also the lack of sustainability of using this limited marine resource for feeding farmed fish (Naylor et al., 2009; Tacon et al., 2011).

The aquaculture industry and scientific community have already shifted towards a lower usage of marine-harvested resources in aquafeed production mainly by introducing vegetable oils (VO), but terrestrial fats could also be sustainable alternatives to fish oil. The inclusion of VO in aquafeeds is one of the most studied alternatives to fish oil on a global level. Marine fish have been reported to have variable responses to dietary incorporation of high amounts of VO that depends not only on the species, but also on the lipid source and incorporation level (Regost et al., 2003; Fountoulaki et al. 2009; Reis et al, 2014). In particular, fish oil substitution by up to 60% VO in European seabass has already been successfully achieved without compromising growth performance and feed efficiency (Montero et al., 2005; Mourente and Bell, 2006). The use of land animal fats has still been poorly explored in aquafeeds (Friesen et al., 2012; Gause and Trushenski, 2013; Emery et al., 2016), and only a few studies have focused on its use in carnivorous marine species, such as yellowtail kingfish (*Seriola lalandi*) and gilthead seabream (*Sparus aurata*; Bowyer et al., 2012; Pérez et al., 2014).

The agro-food industries produce large amounts of animal by-products of terrestrial origin that could be valuable sustainable sources of lipids for aquafeeds. Rendered animal fats are rich in saturated fatty acids (SFA), ranging from 28.5% in poultry fat to 47.5% in beef tallow (Turchini et al., 2009). Monounsaturated fatty acids (MUFA) are also present in high levels in rendered animal fats, which are preferentially β-oxidized in fish over polyunsaturated fatty acids (PUFA; Kiessling and Kiessling, 1993). However, both poultry and mammal fats have a very limited content of PUFA. Beef tallow has less than 4% PUFA, and poultry fat approximately 20% (Turchini et al., 2009). In particular, the level of n−3 long chain polyunsaturated fatty acids (LC-PUFA), characteristic of marine fish oil, crucial for fish health and with high nutritional value to consumers, is extremely limited and commonly reported only

at trace level in land animal fats (Turchini et al., 2009). Reports on the impact of fish oil replacement by animal fats are diverse. Trushenski et al. (2011) observed that a substitution of 50% fish oil by terrestrially derived rendered fats (poultry fat, beef tallow, pork lard or restaurant grease) did not affect growth performance in rainbow trout (*Oncorhynchus mykiss*). Similar results were found for a 75% substitution of fish oil by pork lard in sharpsnout sea bream (*Diplodus puntazzo*) and by poultry fat in sablefish (*Anoplopoma fimbria*; Nogales-Mérida et al., 2011; Friesen et al., 2012). However, a 75% fish oil substitution by chicken fat led to a decline in growth performance in rainbow trout (Turchini et al., 2013) and a decrease in LC-PUFA content in sharpsnout sea bream (Nogales-Mérida et al., 2011). Pérez et al. (2014) observed that, in gilthead seabream (*Sparus aurata*), a complete replacement of fish oil by a mixture of beef tallow and pure linolenic acid-corn oil led to poorer growth performance, as well as significant decreases of muscle and liver EPA + DHA contents. Carnivorous marine species, such as European seabass, have relatively high dietary requirements of n-3 LC-PUFA, namely EPA and DHA, since they generally have low rates of fatty acid bioconversion (Tocher, 2010). Thus, changes in dietary marine ingredients may not only directly modify tissue fatty acid composition, ultimately reducing the nutritional benefits associated with fish consumption (Mourente and Dick, 2002; Mourente et al., 2005), but also compromise farmed fish metabolism, health and performance (Klinger and Naylor, 2012). Due to their lower price and higher availability, land animal fats could be sustainable alternatives to partially replace dietary fish oil (Bureau et al., 2002; Turchini et al., 2009), but their inclusion level should not impair the fish fillet quality or the fish well-being.

The overall aim of this study was to assess the feasibility of replacing fish oil by a mixture of poultry and mammal fats (approximately 70% pork lard and 30% beef tallow) in diets for European seabass juveniles. The effects of this substitution followed a classical zootechnical approach by determining growth performance, feed efficiency, whole-body composition and nutrient digestibility, but also included a specific metabolic evaluation focused on postprandial plasma metabolite, muscle fatty acid profile, lipogenic enzyme activity and liver histologic characterization.

Materials and Methods

Ingredients and Experimental Diets

Table 32. Formulation and proximate composition of experimental diets.

Ingredients (%)	FO	50PFMF	75PFMF	100PFMF
Fish meal 60[1]	22.0	22.0	22.0	22.0
CPSP 90[2]	3.0	3.0	3.0	3.0
Feather meal[3]	5.0	5.0	5.0	5.0
Haemoglobin powder[4]	5.0	5.0	5.0	5.0
Poultry meal 65[5]	20.0	20.0	20.0	20.0
Pea protein concentrate[6]	3.5	3.5	3.5	3.5
Soybean meal 48[7]	9.0	9.0	9.0	9.0
Rapseed meal[8]	3.5	3.5	3.5	3.5
Wheat meal[9]	14.8	14.8	14.8	14.8
Fish oil[10]	12.0	6.0	3.0	
Poultry fat[10]		3.0	4.5	6.0
Mammals fat[10]		3.0	4.5	6.0
Vit & Min Premix PV01[11]	1.0	1.0	1.0	1.0
Lutavit E50[12]	0.03	0.03	0.03	0.03
Choline chloride[12]	0.07	0.07	0.07	0.07
Betaine HCL[12]	0.1	0.1	0.1	0.1
Binder[13]	0.3	0.3	0.3	0.3
Antioxidant[14]	0.2	0.2	0.2	0.2
Sodium propionate[12]	0.1	0.1	0.1	0.1
L-Lysine[15]	0.2	0.2	0.2	0.2
DL-Methionine[16]	0.1	0.1	0.1	0.1
L-Taurine[12]	0.2	0.2	0.2	0.2
Chemical composition				
Dry Matter. DM (%)	94.4	92.6	92.7	92.5
Crude protein (% DM)	50.8	51.3	52.5	51.8
Crude fat (% DM)	18.7	19.5	19.4	19.0
Gross Energy (kJ/g DM)	22.8	23.2	23.3	23.2
Ash (% DM)	10.6	10.7	10.9	10.9
Total P (% DM)	1.4	1.4	1.4	1.3

The abbreviations for the experimental diets stand for: FO – reference diet; 50PFMF. 75PFMF and 100PFMF – diets with 50. 75 and 100% fish oil substitution by a blend (50:50) of poultry and mammal fats, respectively;

[1] Fair Average Quality (FAQ) (62% crude protein, 12% crude fat), COFACO, Portugal;

[2] CPSP90 (84% crude protein, 12% crude fat) SOPROPÊCHE, France;

[3] Feather meal (84.2% crude protein, 10.4% crude fat), SAVINOR SA, Portugal;

[4] Hemoglobin powder (91.6% crude protein, 1.2% crude fat), SONAC BV, The Netherlands;

[5] Poultry meal (69.1% crude protein, 13.7% crude fat), SAVINOR SA, Portugal;

[6] NUTRALYS F85F (78% crude protein, 1% crude fat), ROQUETTE Frères, France;

[7] Dehulled solvent extracted soybean meal (47.7% crude protein, 2.2% crude fat), Cargill, Espanha;

[8] Rapeseed meal (36% crude protein, 2.7% crude fat), PREMIX Lda, Portugal;

[9] Wheat meal (10.2% crude protein, 1.2% crude fat), Casa Lanchinha, Portugal;

[10] SAVINOR SA, Portugal;

[11] Premix for marine fish. PREMIX Lda. Portugal. Vitamins (IU or mg kg-1 diet): DL-alpha tocopherol acetate. 100 mg; sodium menadione bisulphate. 25 mg; retinyl acetate. 20000 IU; DL-cholecalciferol. 2000 IU; thiamin. 30 mg; riboflavin. 30 mg; pyridoxine. 20 mg; cyanocobalamin. 0.1 mg; nicotinic acid. 200 mg; folic acid. 15 mg; ascorbic acid. 1000 mg; inositol. 500 mg; biotin. 3 mg; calcium panthotenate. 100 mg; choline chloride. 1000 mg. betaine. 500 mg. Minerals (g or mg kg-1 diet): cobalt carbonate. 0.65 mg; copper sulphate. 9 mg; ferric sulphate. 6 mg; potassium iodide. 0.5 mg; manganese oxide. 9.6 mg; sodium selenite. 0.01 mg; zinc sulphate.7.5 mg; sodium chloride. 400 mg; calcium carbonate. 1.86 g;

[12] PREMIX Lda, Portugal;

[13] Kieselguhr (natural zeolite), LIGRANA GmbH, Germany;
[14] Paramega PX, KEMIN EUROPE NV, Belgium;
[15] Lysine HCl 99%, Ajinomoto Eurolysine SAS, France;
[16] DL-Metionine 99%: Evonik Degussa GmbH. Germany.

Four extruded isoproteic (51% dry matter - DM) and isoenergetic (23 kJg^{-1} DM) diets were tested in triplicate tanks: a control diet with fish oil as main lipid source (FO) and three diets with substitution of fish oil by a blend (50:50) of poultry and mammal fats at 50, 75 and 100% (50 PFMF, 75PFMF and 100 PFMF). The poultry fat used in this study resulted from poultry by-products rendered by SAVINOR S.A.. The mammal fat consisted in a mixture of lard and beef tallow (approximately 70:30) obtained from pork and beef by-products, also rendered by SAVINOR S.A.. Diets were produced at high temperatures (105–110 °C) through pilot-scale twin-screw extruder (CLEXTRAL BC45, France) with a screw diameter of 55.5 mm, by SPAROS, Lda. (Portugal).

Extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 3 h at 60 °C. The oil fraction was added to cooled pellets (sized 2.0 mm), in a Pegasus vacuum coater (PG-10VCLAB, DINNISEN, The Netherlands). Diets were stored at 4 °C until further use. Ingredient and proximate composition of the diets are presented in Table 32. Fatty acid composition of the diets is presented in Table 33.

Table 33. Fatty acid composition of the experimental diets (%)

	FO	50PFMF	75 PFMF	100 PFMF
C14:0	3.9	2.7	1.9	1.6
C16:0	19.4	20.4	20.2	21.1
C18:0	4.9	6.6	5.2	7.9
Σ SFA [1)]	31.0	32.0	28.8	32.0
C16:1n-7	5.6	4.5	5.0	4.0
C18:1n-9	20.0	26.0	30.1	33.6
C18:1n-7	2.8	2.7	2.3	2.6
C20:1n-9	2.3	1.7	1.4	1.2
C22:1n-11	2.0	1.7	1.5	1.2
Σ MUFA [2)]	34.1	37.8	41.3	43.5
C18:2n-6	8.2	12.5	17.8	16.8
C18:3n-3	1.1	1.2	1.4	1.2
C18:4n-3	1.0	0.8	0.5	0.3
C20:5n-3 (EPA)	5.3	3.4	2.0	0.8
C22:5n-3	1.0	0.6	0.4	0.3
C22:6n-3 (DHA)	9.7	5.5	3.0	1.2
Σ PUFA [3)]	30.9	27.1	27.6	22.5
Σ n-6	11.1	14.5	19.5	18.2
Σ n-3	18.8	12.0	7.7	4.0
n-6/n-3	0.6	1.2	2.6	4.5

1) ΣSFA is the sum of saturated fatty acids and includes also C12:0, C15:0, C17:0
2) ΣMUFA is the sum of mono-unsaturated fatty acids and includes also C16:1, C22:1n-9, C24:1n-9
3) ΣPUFA is the sum of polyunsaturated fatty acids and includes also C16:2n-4, C20:2n-6, C20:4n-6, C21:5n-3
Σn−6 is the sum of n−6 polyunsaturated fatty acids
Σn−3 is the sum of n−3 polyunsaturated fatty acids

Growth Trial

All experiments were performed by trained scientists and according to the Guidelines of the European Union (Directive 2010/63/UE) for the use of laboratory animals. European seabass juveniles were obtained from MARESA (Ayamonte, Spain) and transported to our facilities at CIIMAR, Porto, Portugal. Fish were kept in quarantine for 2 weeks and fed with a commercial diet (AQUASOJA – crude protein: 49% dry matter (DM), crude fat: 20% DM). Subsequently, groups of 50 individuals with an average body weight of 20.3 ± 3.0 g and 12.4 ± 0.6 cm length were randomly distributed among 12 square fiberglass tanks (220 L) in a recirculating water system (4 L min^{-1} flow rate). Each tank was supplied with filtered, heated (21 ± 1 ºC) saltwater (35 ‰). Photoperiod was set to 12L:12D and oxygen concentration was kept above 90% saturation. The pH, ammonia, nitrites and nitrates were regularly monitored and kept at optimal levels for the species. Triplicate groups of fish for each treatment were fed by temporized automatic feeders, until apparent satiation, three times a day (8 h, 12 h and 17 h) for 114 days. In order to regulate the quantity of feed provided daily, the presence or absence of uneaten feed in the tank was checked after each meal and adjustments were made accordingly (Campos et al., 2017).

Sampling

Before the start of the feeding trial, fish were fasted for 24 hours. Fifteen fish from the initial stock were randomly selected, sacrificed by exposure to 140 mg L^{-1} of MS222 solution and stored at −20 °C for determination of initial whole body proximate composition. Throughout the experiment, fish were bulk weighed twice and data on distributed feed and weight gain was gathered. At the end of the trial, fish were fasted for 24 hours and individual weight (g) and fish total length (cm) were recorded. Total feed consumption by each tank was also registered. Six fish from each experimental tank (18 fish per dietary treatment) were randomly selected for blood collection from the caudal vein, using syringes with EDTA. After being transferred into 1.5 mL safe-seal tubes with EDTA, blood was centrifuged (2000 × g for 10 min at 4 °C). Plasma samples were collected and stored at −80 °C for glucose, non-esterified fatty acids, cholesterol and triacylglycerol analysis. Subsequently, the same fish were sacrificed by a sharp blow on the head before collecting viscera and liver, which were weighed to determine the viscerosomatic and hepatosomatic indexes. The liver was stored at −80 ºC for analysis of total lipids and lipogenic enzymatic activity. A small portion of the liver was fixed in phosphate-buffered formalin 4% (pH 7) for 24 h and kept in alcohol 70% until further processing for histologic evaluation. Dorsal muscle was also collected for total lipid and fatty acids analysis. Lastly, 5 fish from each tank were randomly selected, sacrificed as described for the fish from

the initial stock, and stored at −20 °C for the determination of the final whole body proximate composition.

Digestibility Trial

After the growth trial, the remaining fish from each dietary treatment (mean body weight 62.5 ± 12.4 g) were pooled and divided into 3 new homogenous groups for each experimental diet, before being transferred to a new recirculation Guelph System for feces collection. The rearing conditions were the same as described for the growth trial and fish were also fed the same experimental diets, but with 1% of chromium oxide added as an inert marker. Fish were fed once a day, for 5 days before the beginning of feces collection (acclimation period). Feces were then collected every morning from the sedimentation column, before feeding, centrifuged and frozen at −20 ºC, during a period of about 10 days. Around 30 minutes after feeding, each tank was cleaned to ensure that the tanks and sedimentation column were free of uneaten feed. Prior to analysis, feces were freeze-dried.

Feed, Whole body Composition and Feces Analysis

Whole fish from each tank were pooled, ground and moisture content was determined (105 ºC for 24 h). Before further analysis fish were freeze-dried. Feed, feces and whole-body samples were analyzed for dry matter (105 ºC for 24 h), ash by combustion using a muffle furnace (500 ºC for 5 h), crude protein content (N × 6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude fat content by petroleum ether extraction (at 140 °C), in a Soxttherm Multistat/SX PC apparatus (Gerhardt, Germany) and gross energy in an adiabatic bomb calorimetric system (IKA C2000 - IKA-Werke GMBH & CO.KG, Staufen, Germany). Dietary and fecal chromium oxide concentration were determined after perchloric acid digestion, as described by Bolin et al. (1952). Chemical analyses followed AOAC procedures (2006) and were performed in duplicate.

Total Lipids and Fatty Acid analysis

Total lipids were determined gravimetrically according to Folch et al. (1957), replacing chloroform by dichloromethane. The fatty acid methyl esters (FAME) contained in the experimental diets and dorsal muscle were transesterified by acidic methylation (Lepage and Roy, 1984), by adding to each extract 1 mL of anhydrous toluene, 0.6 mg of internal standard solution (1 mL of 0.6 mg C19:0 mL^{-1} hexane; C19:0, Matreya LLC, Pleasant Gap, PA, USA) and 3 mL of freshly prepared 5% (v v^{-1}) acetyl chloride in anhydrous methanol. The mixture was incubated at 100 ºC for 1 hour and cooled at room temperature. FAME were then recovered in 2 mL of hexane. FAME analysis was made through a Shimadzu GC-2010 Plus

gas chromatograph (Shimadzu Europe GmbH, Germany), equipped with a flame-ionization detector (GC-FID) and a Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Supelco, Bellefonte, USA). 1.0 µL helium was injected as a carrier gas at 35 cm s^{-1}, with a split ratio of 1:100. The thermal gradient went from 150 ºC for 7 min, 150 ºC to 170 ºC at 3 ºC min^{-1}, maintained at 170 ºC for 25 min, to 220 ºC at 3 ºC min^{-1} and kept at 220 ºC for 30 min. The injector and detector temperature were maintained at 250 and 260 ºC, respectively. Fatty acids were identified by comparison with known standard mixtures (PUFA-3 Mixture, Bacterial Acid Methyl Esters CP Mixture, and GLC-110 Mixture, Matreya LLC, Pleasant Gap, USA; Supelco 37 Component FAME Mix, Sigma-Aldrich Co. LLC, Bellfonte, USA). FAME present in feed and muscle tissue were expressed as the % of total FAME. To allow FAME quantification as mg g^{-1} sample, 1 mg of Nonadecylic acid (C:19) was added to each sample, during the transesterification process, as an internal standard.

Plasma metabolite assays

Plasma glucose, cholesterol, triacylglycerol and non-esterified fatty acids (NEFA) were determined spectrophotometrically using and following the instructions of commercial kits: Glucose-TR (Spinreact, SA, Spain); Cholesterol (Spinreact, SA, Spain), Triglycerides (Spinreact, SA, Spain) and NEFA (Wako Chemichals, Richmond, VA).

Liver Histological Evaluation

The fixed samples from the liver were dehydrated, embedded in paraffin, and either stained with Haematoxylin and Eosin for cytoplasm vacuolation degree determination, or with Periodic Acid Schiff for glycogen content determination. Observations were made under light microscopy at 100x magnification, for general aspects (Olympus BX51, GmbH, Hamburg, Germany). For grading, at least 20 randomly sampled fields were analyzed per section, at 400× magnification. Hepatocytes vacuolation degree was determined, based on a semi-quantitative approach previously described by Figueiredo-Silva et al. (2005), by assigning one of the following 4 grades: Grade 1 (low) – on average, no more than 1/3 of the hepatocyte cytoplasm shows vacuolation; Grade 2 (moderate) – on average, between 1/3 and 2/3 of the hepatocyte cytoplasm shows vacuolation; Grade 3 (high) – on average, at least 2/3 of the hepatocyte cytoplasm show vacuolation; Grade 4 (extreme) – Virtually total cytoplasm vacuolation. Liver glycogen was assessed as reported by Matos et al. (2013), using a semi-quantitative approach based in three grades: 1 (low) – Hepatocytes presented a weak coloration; 2 (mild) – Hepatocytes presented a mild coloration; 3 (intense) – Hepatocytes presented an intense coloration.

Lipogenic Enzyme Activity Measurements

Liver samples for lipogenic enzyme assays were homogenized in six volumes of ice-cold buffer (100 mM Tris-HCL, 0.1 M EDTA, 0.1% (v v^{-1}) Triton X-100, 50mM PMSF) and centrifuged at 30000 × g, at 4 °C for 30 min. Selected lipogenic enzyme activities were assayed in the supernatant: glucose-6-phosphate dehydrogenase (G6PD) activity was assayed as previously described by Bautista et al. (1988), using a reaction mixture containing 1 M Tris–HCl buffer (pH 7.8), 200 mM $MgCl_2$, 10 mM NADP and 20 mM glucose-6-phosphate. Malic enzyme (ME) activity was assayed according to Ochoa (1955), using a reaction mixture containing 74.5 mM imidazole–HCl buffer (pH 7.4), 100 mM $MgCl_2$, 8 mM NADP and 40 mM L-malate. Fatty acid synthase (FAS) activity was assayed as previously described by Chang et al. (1967), modified by Chakrabarty & Leveille (1969). A reaction mixture of 100 mM potassium phosphate buffer (pH 6.5), 0.1 mM NADPH and 25 µM acetyl-CoA and 600 mM malonyl-CoA was used. Enzyme activity was determined at 37 °C and the changes in absorbance were monitored using a microplate reader (ELx808; Bio-Tek Instruments, Winooski, Vermont, USA). All enzyme activities were expressed as specific activity (milliunits per mg of hepatic soluble protein). Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard. One unit of enzyme activity was defined as the amount of enzyme necessary to convert 1 µmol of substrate at assay temperature.

Calculations

The apparent digestibility coefficients (ADCs) of the experimental diet were calculated according to Maynard et al. (1979): Dry matter ADC (%) = 100 × (1 – (dietary Cr_2O_3 level / feces Cr_2O_3 level); nutrients and energy ADC (%) = 100 × (1 – (dietary Cr_2O_3 level / feces Cr_2O_3 level) × (feces nutrient or energy level / dietary nutrient or energy level).

Growth parameters and feed utilization were calculated as follows: Daily growth index (DGI; g/day) = (($Wf^{1/3}$ – $Wi^{1/3}$) / days) x 100, where Wi and Wf are the initial and final weights; Feed conversion ratio (FCR) = dry feed intake / (Wf – Wi); Protein efficiency ratio (PER) = (Wf – Wi) / total protein intake (g). Nutrient and energy intake (g or kJ/kg ABW/day) = nutrient intake × diet nutrient content (%DM), where the average body weight (ABW, g) is the ratio (Wi + Wf) / 2. Nutrient and energy gain (g or kJ/kg ABW/day) = (Final carcass nutrient content – initial carcass nutrient content) / ABW / days; Nutrient and energy retention (% of digestible intake) = 100 × (final weight × final carcass nutrient content – initial weight × initial carcass nutrient content)/ (intake × diet nutrient content (%DM) × nutrient ADC).

Somatic indices were calculated to assess the condition of animals at the end of the experiment: VSI (%) = 100 x visceral weight (g) / Wf (g); HSI (%) = 100 x liver weight (g) / Wf (g).

Statistical Analysis

Statistical analyses followed the methods outlined by Zar (1999). Data were tested for normality and homogeneity of variances using Kolmogorov – Smirnov and Levene's test, respectively. Then data were analyzed with one-way analysis of variance (ANOVA) to test differences between dietary treatments. When this test showed significance, individual means were compared using the Tukey HSD test. Significant differences were considered when $P < 0.05$. When ANOVA criteria were not fulfilled, data were submitted to the nonparametric tests (Kruskal – Wallis test), followed by Mann – Whitney test whenever needed. All statistical tests were performed using the program IBM SPSS, 21.0, IBM Corporation (2011).

Results

Growth performance and feed utilization

After the 114 days, all groups of fish tripled their initial body weight (Table 34). In the present study, FCR, DGI and PER were not significantly different among dietary treatments. Likewise, nutrient intake was similar in all groups (Table 34). However, final body weight and final body length were significantly lower in fish fed 100PFMF (60 g) than in fish fed the remaining diets (63 – 64 g).

Table 34. Growth performance and feed intake of European seabass fed the experimental diets for 114 days.

	FO	50PFMF	75PFMF	100PFMF
Initial Body Weight (g)	20.3 ± 3.1	20.3 ± 3.1	20.3 ± 3.0	20.3 ± 3.0
Final Body Weight (g)	63.7 ± 13.6[a]	63.3 ± 10.9[a]	63.5 ± 11.8[a]	59.6 ± 12.8[b]
Body Length (cm)	17.8 ± 1.3[a]	17.8 ± 1.0[a]	17.8 ± 1.1[a]	17.5 ± 1.3[b]
DGI	1.11 ± 0.01	1.10 ± 0.06	1.11 ± 0.01	1.03 ± 0.01
FCR	1.47 ± 0.04	1.49 ± 0.11	1.44 ± 0.17	1.56 ± 0.02
PER	1.34 ± 0.03	1.32 ± 0.10	1.33 ± 0.15	1.23 ± 0.02
Intake (g or kJ kg^{-1} ABW day^{-1})				
Dry matter	13.3 ± 0.3	13.4 ± 1.2	13.0 ± 1.4	13.5 ± 0.3
Protein	6.8 ± 0.2	6.9 ± 0.6	6.8 ± 0.7	7.0 ± 0.1
Lipid	2.5 ± 0.1	2.6 ± 0.2	2.5 ± 0.3	2.6 ± 0.1
Energy	302.8 ± 7.0	311.2 ± 27. 2	302.2 ± 31.9	313.5 ± 5.8

DGI: Daily growth index; FCR: Feed conversion ratio; PER: Protein efficiency ratio; ABW: Average body weight

Values represent mean ± standard deviation. In each row, different letters indicate significant differences between treatments.

Fish composition and nutrient utilization

The viscerosomatic index remained unaffected by the increasing level of dietary animal fat (Table 35). However, in fish fed 100PFMF, the hepatosomatic index was significantly increased (1.3) compared to all other treatments (1.0 – 1.1), and the liver total lipid content followed the same trend (29% in 100PFMF *vs* 21-26% in other dietary treatments). Muscle lipid content did not vary among groups and whole-body composition also remained similar among groups. Protein and energy apparent digestibility coefficients (ADC; Table 36) were not affected by the dietary treatments (90 and 84 – 85%, respectively), but lipid ADC was lowest in fish fed 100PFMF. Nitrogen and energy balance remained unaffected by the dietary treatments (Table 36).

Table 35. Somatic indexes, tissue lipid content and whole-body composition of European seabass fed the experimental diets for 114 days.

	FO	50PFMF	75PFMF	100PFMF
HSI	1.1 ± 0.2[b]	1.1 ± 0.2[b]	1.0 ± 0.2[b]	1.3 ± 0.3[a]
VSI	5.3 ± 0.6	5.2 ± 1.2	5.2 ± 1.1	5.1 ± 0.6
Muscle total lipids (% WW)	2.3 ± 0.1	1.9 ± 0.4	1.9 ± 0.1	2.3 ± 0.1
Liver total lipids (% WW)	20.8 ± 5.0[b]	23.3 ± 1.6[b]	25.6 ± 2.9[b]	28.9 ± 2.6[a]
Whole-body Composition (mg g^{-1} or kJ g^{-1} WW)				
DM	34.6 ± 0.8	34.3 ± 0.7	34.3 ± 0.5	35.3 ± 0.3
Ash	4.0 ± 0.4	4.3 ± 0.1	4.3 ± 0.1	4.5 ± 0.3
Protein	17.3 ± 0.4	17.6 ± 0.2	17.5 ± 0.2	16.9 ± 0.4
Lipids	13.3 ± 1.0	12.6 ± 0.1	13.0 ± 0.6	14.4 ± 0.4
Energy	8.9 ± 0.3	8.8 ± 0.1	8.8 ± 0.2	9.2 ± 0.2

HSI: Hepatosomatic index; VSI: Viscerosomatic index; DM: Dry matter; WW: Wet weight. Values represent mean ± standard deviation. In each row, different letters indicate significant differences between treatments

Table 36. Apparent digestibility coefficient (ADC) of macronutrients and balance of nitrogen and energy of European seabass fed the experimental diets for 114 days.

	FO	50PFMF	75PFMF	100PFMF
ADC (%)				
Dry Matter	75.6 ± 0.9	74.4 ± 1.3	75.8 ± 0.1	76.1 ± 1.4
Protein	89.9 ± 0.3	89.9 ± 0.3	90.3 ± 0.2	90.1 ± 1.5
Lipid	95.4 ± 0.1[a]	93.9 ± 0.4[a]	94.5 ± 0.2[a]	92.8 ± 1.5[b]
Energy	84.9 ± 0.3	83.5 ± 0.4	85.0 ± 0.5	84.5 ± 1.8
N balance (g kg-1 ABW day-1)				
Digestible N intake (DN)	0.97 ± 0.02	0.99 ± 0.09	0.99 ± 0.10	1.01 ± 0.002
N gain	0.26 ± 0.01	0.26 ± 0.01	0.259 ± 0.002	0.24 ± 0.01
N retention efficiency (% DN)	26.3 ± 0.5	26.4 ± 2.2	26.5 ± 2.9	23.4 ± 0.8
Faecal N losses	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
Metabolic N losses	0.72 ± 0.02	0.73 ± 0.08	0.73 ± 0.11	0.77 ± 0.01
Energy balance (kJ kg-1 ABW day-1)				
DE intake	256.9 ± 5.3	259.9 ± 23.3	257.1 ± 28.7	264.9 ± 1.0
E gain	78.7 ± 3.5	76.5 ± 3.9	77.0 ± 2.7	78.3 ± 2.4
Faecal E losses	45.9 ± 1.8	51.3 ± 4.1	45.1 ± 3.2	48.6 ± 6.6
Metabolizable E	178.3 ± 7.7	183.4 ± 22.9	180.1 ± 27.4	186.6 ± 3.4
Heat production	224.1 ± 9.0	234.7 ± 27.0	225.2 ± 30.5	235.2 ± 3.7

Plasma Metabolites

Seabass plasma metabolites concentrations are presented in Fig. 1a, b. Plasma glucose, non-esterified fatty acids (NEFA), cholesterol (CHOL), and triacylglycerol (TAG) were not significantly affected by the inclusion of terrestrial animal fats.

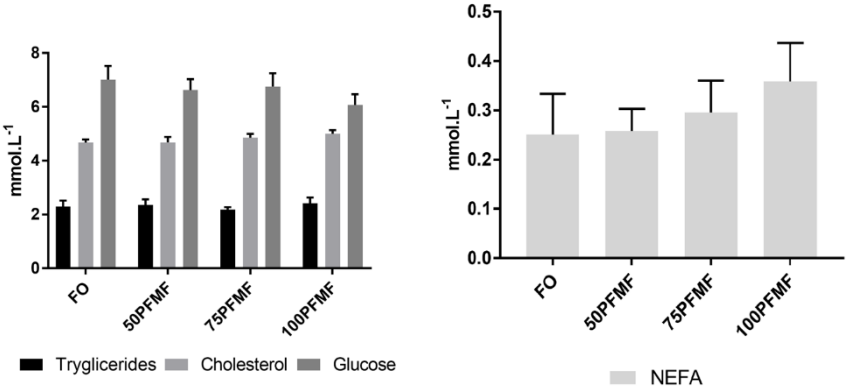

Figure 15. Plasma Triglycerides, Glucose, Cholesterol and NEFA concentrations (mmol L^{-1}) in seabass fed the experimental diets and after a fasting period of 24 h. The values are means ± SD, n = 18.

Lipid content and Muscle fatty acid composition

Muscle fatty acid profile was determined by GC-FID (Table 37). The replacement of fish oil by animal fat in seabass diets significantly affected fish muscle fatty acid profile. Although total saturated fatty acids were not affected by the dietary treatments, the C18:0 significantly increased whereas C14:0 decreased with fish oil reduction. Muscle monounsaturated fatty acid levels increased significantly with the degree of fish oil substitution above 50%, with higher levels being found in fish fed100PFMF, mainly due to increased levels of oleic acid (18:1n-9). The polyunsaturated fatty acids decreased significantly with higher levels of fish oil substitution (> 50%). Linoleic acid (18:2n−6) deposition increased concomitantly with dietary animal fat level, whereas alpha-linolenic acid (18:3n−3) content increased significantly in fish fed 100PFMF compared to the FO fed fish. Although higher levels of n-3 PUFA were systematically found in fish flesh than in the experimental diets, due to an accumulation of eicosapentaenoic (EPA; 20:5n-3) and especially docosahexaenoic (DHA; 22:6n-3) fatty acids, muscle content of EPA and DHA decreased concurrently with decreasing dietary fish oil levels; fish fed 100PFMF had the lowest levels of EPA and DHA (Table 37). DHA/EPA was not affected by the dietary treatments, but fish fed FO showed a significantly lower n6/n3 ratio in muscle than those fed the remaining diets (Table 37). The level of EPA + DHA per 100 g of muscle was significantly higher in fish fed FO (0.5 g 100 g^{-1}) than in fish fed the remaining diets (0.3 – 0.4 g 100 g^{-1}). The atherogenicity index was not affected by the

experimental diets, but a significant increase in the thrombogenicity was observed with 75% and complete fish oil replacement, from 0.19 to 0.41 and 0.63, respectively (Table 37).

Table 37. Muscle fatty acid profile (g/100 g total fatty acids) of European seabass fed the experimental diets for 114 days.

	FO	50PFMF	75PFMF	100PFMF
C14:0	1.9 ± 0.1^{a}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	1.2 ± 0.1^{b}
C16:0	17.2 ± 0.3^{b}	17.8 ± 0.1^{b}	18.1 ± 0.1^{b}	18.4 ± 0.3^{a}
C18:0	4.8 ± 0.1^{b}	5.4 ± 0.2^{a}	5.6 ± 0.1^{a}	5.5 ± 0.2^{a}
Σ SFA[1]	25.6 ± 0.4	26.0 ± 0.2	26.3 ± 0.3	26.1 ± 0.6
C16:1*n*-7	3.3 ± 0.2	2.9 ± 0.2	3.0 ± 0.2	3.1 ± 0.1
C18:1*n*-9	20.5 ± 0.1^{b}	24.6 ± 1.3^{ab}	27.9 ± 1.3^{ab}	31.3 ± 1.7^{a}
C18:1*n*-7	2.3 ± 0.03	2.31 ± 0.08	2.31 ± 0.07	2.34 ± 0.06
C20:1*n*-9	1.8 ± 0.1^{a}	1.6 ± 0.1^{b}	1.5 ± 0.1^{b}	1.6 ± 0.1^{ab}
Σ MUFA[2]	29.6 ± 0.4^{c}	32.9 ± 1.4^{cb}	36.3 ± 1.4^{ba}	39.6 ± 1.9^{a}
C18:2*n*-6	8.0 ± 0.2^{d}	11.3 ± 0.7^{c}	13.7 ± 0.4^{b}	16.8 ± 0.3^{a}
C18:3*n*-3	1.25 ± 0.04^{b}	1.34 ± 0.11^{ab}	1.44 ± 0.04^{ab}	1.52 ± 0.1^{a}
C18:4*n*-3	0.56 ± 0.05^{a}	0.45 ± 0.03^{b}	0.40 ± 0.03^{bc}	0.31 ± 0.02^{c}
C20:5*n*-3 (EPA)	5.5 ± 0.2^{a}	4.4 ± 0.2^{b}	3.3 ± 0.2^{c}	1.9 ± 0.2^{d}
C22:5*n*-3	1.30 ± 0.02^{a}	0.99 ± 0.04^{b}	0.87 ± 0.08^{bc}	0.77 ± 0.08^{c}
C22:6*n*-3 (DHA)	19.6 ± 0.5^{a}	15.2 ± 1.5^{b}	10.9 ± 1.5^{c}	6.6 ± 0.9^{d}
Σ PUFA[3]	41.3 ± 0.4^{a}	38.2 ± 1.1^{a}	34.7 ± 1.6^{b}	31.8 ± 1.5^{b}
Σ *n*-6	11.8 ± 0.2^{a}	14.7 ± 0.5^{b}	16.8 ± 0.3^{c}	19.9 ± 0.5^{d}
Σ *n*-3	28.8 ± 0.5^{a}	22.9 ± 1.6^{b}	17.4 ± 1.7^{c}	11.5 ± 1.0^{d}
n-6/*n*-3	0.41 ± 0.01^{d}	0.64 ± 0.06^{c}	0.97 ± 0.10^{b}	1.74 ± 0.12^{a}
DHA/EPA	3.6 ± 0.1	3.4 ± 0.2	3.3 ± 0.2	3.4 ± 0.3
TI[4]	0.191 ± 0.003^{c}	0.28 ± 0.03^{bc}	0.41 ± 0.05^{b}	0.63 ± 0.08^{a}
AI[5]	0.42 ± 0.01	0.411 ± 0.002	0.41 ± 0.01	0.40 ± 0.01
EPA+DHA (g per 100 g of muscle)	0.48 ± 0.07^{a}	0.36 ± 0.03^{b}	0.33 ± 0.01^{b}	0.27 ± 0.03^{c}

Values represent mean ± standard deviation. In each row, different letters indicate significant differences between treatments.
1) ΣSFA is the sum of saturated fatty acids and includes also C12:0, C15:0, C17:0
2) ΣMUFA is the sum of mono-unsaturated fatty acids and includes also C16:1, C22:1*n*-9, C24:1*n*-9
3) ΣPUFA is the sum of polyunsaturated fatty acids and includes also C16:2*n*-4, C20:2*n*-6, C20:4*n*-6, C21:5*n*-3
Σ*n*−6 is the sum of *n*−6 polyunsaturated fatty acids; Σ*n*−3 is the sum of *n*−3 polyunsaturated fatty acids
4) TI is the thrombogenicity index; 5) AI is the atherogenicity index

Liver histology and Lipogenic Enzymes' Activity

The histological appearance of the fish liver sections from fish fed the experimental diets is depicted in Figure 16 and Figure 17. The livers of fish fed FO, 50PFMF and 75PFMF displayed regular-shaped hepatocytes and centrally located cell nuclei (Figure 16). However, significant differences were observed among the different diets regarding glycogen deposition, with the highest level of glycogen being found in the fish fed FO, and the lowest in fish fed 100PFMF (Figure 16; Table 38). Also, a complete substitution of fish oil by PFMF resulted in a significantly higher vacuolation in the hepatocytes (Table 38) and apparent hepatic steatosis (Figure 16). The integrity of the hepatocytes was compromised in fish fed 100PFMF, with obvious swelling nuclei displacement in livers.

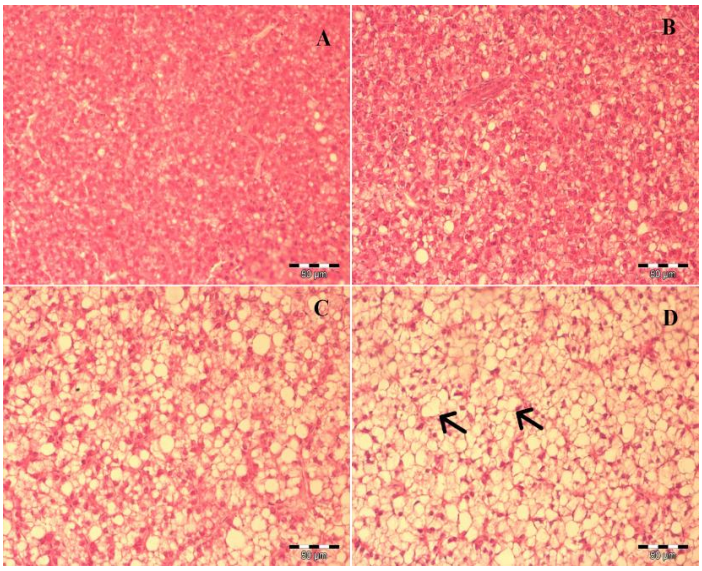

Figure 16. Liver cross-sectional section from seabass fed diet A) FO, B) 50PFMF, C) 75PFMF and D) 100PFMF (0, 50, 75 and 100% substitution, respectively) (H&E, 400). Large vacuoles are indicated by black arrows.

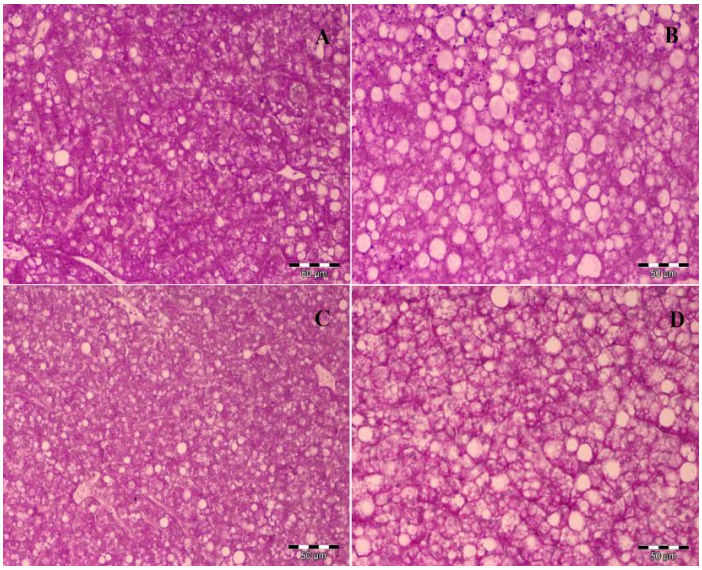

Figure 17. Liver cross-sectional section from seabass fed diet A) FO, B) 50PFMF, C) 75PFMF and D) 100PFMF (0, 50, 75 and 100% substitution, respectively) (PAS, 400).

Data on the activities of the lipogenic enzymes assayed in European seabass liver are reported as mIU per mg protein (Table 38). Fish oil replacement did not affect G6PD activity. However, malic enzyme activity was significantly higher in fish fed FO than in those fed 100PFMF, while FAS activity was significantly lower in fish fed 50PFMF than in fish fed FO and 100PFMF.

Table 38. Vacuolization and glycogen deposition in hepatocytes (n=6) and enzymatic activity (mU/mg protein) of selected enzymes involved in lipogenesis (n=9) in European seabass fed the experimental diets for 114 days.

	FO	50PFMF	75PFMF	100PFMF
Vacuolation	2.2 ± 0.2^{b}	2.2 ± 0.1^{b}	2.5 ± 0.5^{b}	3.3 ± 0.1^{a}
Glycogen	2.2 ± 0.3^{a}	1.7 ± 0.2^{b}	1.7 ± 0.1^{b}	1.4 ± 0.1^{c}
ME	2.1 ± 0.1^{a}	1.5 ± 0.1^{ab}	1.6 ± 0.1^{ab}	1.3 ± 0.1 ^{b}
G6PD	97.7 ± 5.3	80.0 ± 5.5	78.7 ± 6.6	79.1 ± 8.6
FAS	1.1 ± 0.4^{a}	0.7 ± 0.4^{b}	0.9 ± 0.3^{ab}	1.0 ± 0.4^{a}

Values represent mean ± standard deviation. In each row, different letters indicate significant differences between treatments.

Discussion

In the aquaculture industry, intensive research efforts have been made towards replacing fish oil by sustainable ingredients. In this study, total replacement of fish oil by land animal fats in seabass diets led to a significant decrease of fish final body weight, but feed efficiency was not affected. These results are in line with previous studies with sablefish (*Anoplopoma fimbria)* that reported no differences in growth parameters and whole-body composition, when fish oil was replaced up to 75% by animal-derived fats (Friesen et al., 2012). Nogales-Mérida et al. (2011) also reported a replacement of fish oil by pork fat up to 75% with no differences in growth performance parameters, including final body weight and FCR, in sharpsnout sea bream (*Diplodus puntazzo*). Contrarily, in gilthead seabream (*Sparus aurata*), a complete replacement of dietary fish oil by a mixture of beef tallow and pure linolenic acid-corn oil has been associated with poorer growth performance (Pérez et al., 2014). In the present study, nutrient intake was not affected by dietary treatment. This is supported by previous studies in Japanese seabass and yellowtail kingfish, which reported no effect on feed intake when fish oil was partially replaced by animal fats such as beef tallow and poultry fat (Xue et al., 2006; Bowyer et al., 2012).

In the present study, protein and energy digestibility were not affected by dietary treatment. However, total fish oil replacement led to a significantly lower ability to digest dietary lipids. Lipid digestibility is majorly influenced by the lipid source, namely by its fatty acid profile, the fatty acids' degree of saturation, chain length, and the subsequent melting point of the fat. Given the high levels of SFA and MUFA in rendered animal fats, these have higher melting points than fish oil, granting these fats a solid state at environmental temperature (Bureau, et al., 2002). Conversely, under the same conditions, fish and vegetable oils, which are richer in PUFA, are usually liquid. Therefore, the higher content of mammal and poultry fats in the 100PFMF diet could have decreased the digestibility and absorption of the lipids, as these fats provide a smaller surface area available for chemical and enzymatic digestive processes (Bureau and Meeker, 2011). Additionally, relative amounts of SFA, MUFA and PUFA are of major influence in the digestibility of a dietary lipid source. The extreme substitution of fish oil in 100PFMF, and associated lower PUFA content, seem to have a pronounced negative effect in the lipid ADC. However, the fish oil inclusion level, 6% and 3% in 50PFMF and 75PFMF diets, respectively, might have enhanced the digestibility of SFA rich fats present in these diets, resulting in ADC values similar to those observed in fish fed the FO diet.

Although plasma metabolites levels were found consistent with those reported for European seabass (Figueiredo-Silva et al., 2005; Messina et al., 2012; Castro et al., 2015), an increasing trend in non-esterified fatty acids (NEFA) concentration is observed with increased

fish oil replacement. Higher plasmatic NEFA concentrations could have resulted from a mobilization of lipids, stored in the liver, through the circulation and into the working muscles for further use. (McClellan et al., 1995). In fact, as shown in Table 35, regardless of dietary treatment, total lipid content was higher in the liver than in the dorsal muscle. Additionally, liver total lipids increased significantly in fish fed 100PFMF, which could have led to a higher mobilization of lipids into circulation and ultimately into the working muscles. No significant effects were found on circulating levels of cholesterol or triglycerides in seabass. Similarly, Bowyer et al. (2012) reported no differences in cholesterol or triglycerides in yellowtail kingfish (*Seriola lalandi*) when 50% dietary fish oil was replaced by poultry oil. However, a complete fish oil replacement by poultry fat decreased significantly the plasma cholesterol levels in the same species. Pérez et al. (2014) also described a decrease in plasma triglycerides and cholesterol levels in gilthead seabream fed a diet devoid of fish oil, which was replaced by a blend of beef tallow and vegetable oils.

The hepatosomatic index (HSI) showed a significant increase in fish fed 100PFMF when compared to fish fed the remaining diets, displaying a tendency for HSI increase when fish oil was replaced by other lipid sources, as shown in previous studies (Fountoulaki et al., 2009; Bowyer et al., 2012; Borges et al., 2014). Increased HSI is mainly a result of increased lipid deposition in liver, since it is the major lipid storage organ (21 – 29% WW), with fish fed 100PFMF showing the highest liver lipid content that resulted in histomorphological alterations, including hepatic steatosis. In previous studies, such morphologic alterations have been associated with a range of 60 – 69% fish oil replacement by a mixture of vegetable oils (palm, linseed and rapeseed or palm, soybean and rapeseed; Mourente et al., 2005; Fountoulaki et al., 2009), but it seems to be nonpathological and reversible when fish are re-fed with a 100% FO diet balanced diet (Caballero et al. 2004). However, Figueiredo-Silva et al. (2005) reported that up to 50% fish oil replacement by soybean oil did not induce significant alterations in vacuolation degree or glycogen content. Lipid ADC was found to be negative correlated with vacuolation degree ($r = -0.647$) and positively correlated with glycogen deposition ($r = 0.620$): a higher degree of fish oil replacement impaired lipid ADC and led to a higher lipid deposition, particularly in the liver. Moreover, higher hepatic lipid deposition in the 100PFMF group could be due to lower fatty acid oxidation. Dietary n-3 LC-PUFA are responsible for increasing mitochondrial fatty acid oxidation by stimulating the activity of Carnitine Palmitoyl Transferase (CPT I) (Madsen et al., 1999). Thus, a lower expression of CPT I could have led to lower NEFA delivery to the liver, decreasing FA β-oxidation and eventually resulting in higher hepatic lipid deposition. In addition, higher lipid content in the liver could be related to lower dietary n-3 LC-PUFA content, such as EPA, in the 100PFMF group. In fact, it has been reported that EPA has a higher binding affinity to Peroxisome Proliferator-Activated Receptor α (PPARα) than SFA,

MUFA and n-6 PUFA (Desvergne and Wahli, 1999). The decreased PPARα activation by n-3 LC-PUFA may lead to the decreased expression of lipolytic genes, and activity of enzymes such as CPT I, and ultimately a fatty liver (Leone et al., 1999; Leaver et al., 2008). Several authors have reported a fatty liver as a result of high rates of fish oil substitution by alternative lipid sources such as vegetable oils (Caballero et al., 2004; Menoyo et al., 2004) or rendered animal fats (Xue et al., 2006), although their effect in the correct functioning of the liver and its possible reversibility is not well understood. In this study, activities of hepatic lipogenic enzymes in European seabass were generally consistent with previous studies on the same species (Dias et al., 1998, 2005). G6PD activity was systematically higher than ME activity, as previously reported for this species (Castro et al., 2015; Guerreiro et al., 2015) and other fish such as rainbow trout and gilthead seabream (Richard et al. 2006; Bouraoui et al., 2011). This confirms that NADPH is mainly provided by the pentose phosphate pathway. ME activity was significantly reduced with an increased fish oil substitution, whereas FAS activity did not reveal any clear trend. On the other hand, although G6PD activity was not significantly affected by fish oil replacement, a decrease in activity can be observed in fish fed land animal fats. The effects of fish oil replacement in lipogenic enzymes' activity seem to be quite variable. An inhibitory effect on hepatic lipogenic activity has been reported in several fish species by Menoyo et al. (2004) when fish oil was replaced by linseed and soybean oil. However, several authors observed no significant differences in hepatic lipogenesis when fish oil was replaced by linseed and soybean oils in turbot (Regost et al., 2001), and by linseed, palm oil and rapeseed oil both in European seabass (Richard et al., 2006) and in gilthead seabream (Bouraoui et al., 2011). A fatty liver could have impaired function, including lipogenesis enzymatic activity, as reported by Boujard et al. (2004). Nonetheless, the fatty liver and associated metabolic and histomorphological alterations observed in this study are presumed to be related to differences in dietary lipid source. Based on survival, growth, feed conversion, absence of disease, and overall general fish sanitary status, this fatty liver condition did not appear to have adverse effects.

Muscle total lipid content did not vary between groups. In general, fatty acid (FA) composition observed in seabass muscle reflected the dietary fatty acid composition (Table 33). After 114 days of feeding, muscle DHA and EPA contents (DHA: 6.6 – 19.6%; EPA: 1.9 – 5.5%) were higher than the respective dietary level (DHA: 1.2 – 9.6%; EPA: 0.8 – 5.3%) which suggests that these FAs are selectively retained in the muscle. Similarly, previous studies reported DHA retention in muscle of turbot (Regost et al., 2003), Senegalese sole (Borges et al., 2014) and European seabass (Mourente and Bell, 2006; Montero et al., 2005) fed alternative lipid sources. Linoleic (18:2*n*-6) and alpha-linolenic acid (18:3*n*-3) dietary levels were higher than those found in muscle, suggesting that they were preferentially used for

energy or converted to longer chain unsaturated FA. Former studies point to a substrate preference for saturated fatty acids (SFA) in β-oxidation, but this depends on FA availability (Tocher, 2003; Turchini et al., 2009). Fish consumption, in general, is associated with health benefits due to its high levels of n-3 PUFA. These FA have been proven to prevent cardiovascular and neurological diseases (Lavie et al., 2009; Calon et al., 2007) and are a quality trait searched by consumers. Replacement of 50% dietary fish oil reduced n-3 PUFA muscle contents by 8%, a 75% replacement reduced them by 16%, whereas complete replacement reduced them by 23%, when compared to fish fed with the control diet. Reduction of these fatty acids was less pronounced for EPA (19%, 38% and 65% for fish fed 50PFMF 75PFMF and 100PFMF) than for DHA (22%, 44% and 66% for fish fed 50PFMF 75PFMF and 100PFMF; Table 37). As a consequence of such difference in fatty acid utilization, EPA/DHA values were not significantly affected by the inclusion of animal fats of terrestrial origin in the diets. Despite the significant reduction of EPA and DHA levels with increased fish oil replacement, at the end of the trial EPA + DHA content of fish muscle (0.3 – 0.5 g per 100 g) are still above the recommended daily intake (RDI) of EPA + DHA (0.25 g per day for healthy human individuals; Aranceta and Pérez-Rodrigo, 2012). In addition, the lower levels of *n*-3 and *n*-6 fatty acids found in fish fed PFMF suggest that the muscle will be less prone to rancidity, thus delaying wastage and extending shelf-life. To ensure the nutritional quality of the fillet, atherogenicity (AI) and thrombogenicity (TI) indexes were calculated. AI indicates the relationship between the sum of the main SFA and the sum of the main classes of PUFA, the first being considered pro-atherogenic (favor lipid adhesion to cells of the circulatory and immunological systems), and the latter anti-atherogenic (inhibit the accumulation of plaque and decreases the phospholipids, cholesterol, and esterified fatty acids levels, and consequently prevents the development of coronary diseases). On the other hand, TI demonstrates the predisposition for clot formation in the blood vessels. It is defined as the relationship between the pro-thrombogenic (SFA) and the anti-thrombogenetic fatty acids (MUFA, *n*-6 PUFA and *n*-3 PUFA; Ulbricht and Southgate, 1991). In this study, AI was not affected by the experimental diets (0.40 – 0.42), while a significant increase in the TI was observed with 75% and complete fish oil replacement. Higher AI and TI values (> 1.0) are detrimental to human health (Bobe et al., 2004). While there were significant differences in the TI of muscle from seabass fed 75% or total fish oil replacement (0.19 *vs* 0.41 and 0.63, respectively), these values did not exceed 0.6. Therefore, the differences in potential health impact of fillets from fish fed fish oil-based diet or diets with PFMF were minor. Turchini et al. (2003) have reported similar results, with no significant differences on AI but significantly increased TI, from 0.25 to 0.31 and 0.34, when fish oil was replaced by poultry fat or pork lard, respectively.

Conclusions

Juvenile seabass can effectively use diets with high levels of a blend of poultry and mammal fat, as alternative lipid source, replacing up to 75% fish oil without impairing feed intake, growth performance and nutrient utilization. Despite alteration of the muscle FA profile, European seabass still provides the recommended daily intake levels of EPA + DHA (0.3 – 0.5 g per 100 g of muscle) and presents low levels of atherogenicity (AI) and thrombogenicity (TI) indexes (< 1). Thus, European seabass fed with up to 75% land animal fats as an alternative to fish oil, can still be considered a good nutritional value end-product for human consumption.

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Chapter VII

Life-cycle assessment of animal feeds from poultry by-products: poultry fat, poultry by-product meal and hydrolyzed feather meal

Life-cycle assessment of animal feeds from poultry by-products: poultry fat, poultry by-product meal and hydrolyzed feather meal

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Abstract

This study presents an environmental life cycle assessment of poultry fat (PF), poultry by-product meal (PBM) and steam hydrolyzed feather meal (HF), produced from rendering poultry by-products. By rendering these poultry by-products, valuable nutrient sources can be obtained, which can be used as feedstuffs for the fish feed industry, displacing traditional fishmeal and fish oil, promoting a circular economy system by reducing waste and adding value to by-products of the poultry production and reintroducing them in the economy as high-value products. A life cycle inventory and model were implemented for the HF, PBM and PF production chains based on data collected from two poultry by-product rendering units. Four environmental impact categories have been assessed using the CML life-cycle impact assessment method: global warming (GW), abiotic depletion (AD), acidification (AC) and eutrophication (EUT). The results show that the poultry production is the main contributor for impacts in AC and EUT, while the rendering process of the by-products is the main responsible for GW and AD impacts, mainly due to heat generation. A sensitivity analysis was conducted to assess changing the type of fuel from fuel oil to wood pellets used for heat generation in the rendering process, aiming at reducing impacts. The results show that using wood pellets reduces environmental impacts (except for eutrophication). A sensitivity analysis of alternative multifunctionality procedures for dealing with poultry by-products was performed, showing that the selected allocation method has a huge influence on the life cycle impacts, with higher impacts when mass allocation is employed instead of economic allocation. Overall, the production of 1 t of PF, PBM and HF from poultry by-products generates 666, 726 and 597 kg CO_2 (GW), 4.2, 4.6 and 4.7 kg SO_2 (AC), 1.8, 2.0 and 2.1 kg PO_4^{3-} (EUT), and 3.8, 4.2 and 3.2 kg Sb (AD), respectively. The relative low impact of such ingredients compared with fishmeal and fish oil indicates that using them as fishmeal and fish oil replacers would decrease substantially the environmental impacts of animal feed production in the impact categories considered.

Introduction

The Food and Agriculture Organization (FAO) of the United Nations estimates that over 1.3 billion tons of food are wasted every year (FAO, 2011). Meanwhile, the fast world population growth over the last 50 years has triggered a huge intensification in food demand. This growth will require a 60% – 70% increase in food production by 2050 (Moraes et al., 2014), which will further increase the amounts of waste if actions are not taken. Big amounts of resources channeled into food production are not only being lost but also contributing to generate environmental impacts, namely by increasing greenhouse gas emissions (FAO, 2011). Moreover, recently the concept of circular economy has been strongly promoted as a mean to reduce waste by adding value to by-products and reintroducing them in the economy for as long as possible (European Commission, 2014).

The production of poultry for Human consumption, for instance, is responsible for the generation of large amounts of by-products. In the European Union alone, every year, 6 billion chickens, turkeys and other poultry are slaughtered for meat production and about 25% of each of these animals is not used for direct Human consumption (EPFRA, 2016). By rendering these poultry by-products, valuable nutrient sources can be obtained, namely poultry by-product meal (PBM; obtained from heads, bruised meat, bones and viscera) and hydrolyzed feather meal (HF; obtained from feathers and sometimes together with some blood), interesting sources of protein, antioxidants and fatty acids (Lasekan et al., 2013). Poultry fat (PF) is also obtained from the heads, bruised meat, bones and viscera of poultry. PF is richer in monounsaturated fatty acids (MUFA) than in saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), being particularly rich in n-6 PUFA and poor in n-3 PUFA (Emery et al., 2014). Although PF lacks important n-3 PUFA, such as EPA and DHA, the lower PUFA and higher MUFA content of PF makes it a more stable ingredient to be included in animal feeds than n-3 PUFA rich sources like fish oil (Watanabe, 1989).

The re-approval of non-ruminant land animal meals in European aquafeeds (European Commission, 2013) has sparked the interest in searching for nutrient sources in land animal by-products, which are widely generated and can be valuable biological resources (NRC, 2011). Even though using these by-products in feeds seems to be beneficial, their collection and valorization processes generate environmental impacts, which need to be evaluated through a life cycle perspective to quantify the potential benefits of using these ingredients as alternatives to the traditional fishmeal and fish oil used in fish feeds.

Life cycle assessment (LCA) studies have been implemented to assess the valorization of by-products from various food production sectors such as mussel production (Iribarren et

al., 2010), canning industry (Laso et al., 2016) and human food preparation (Ogino et al., 2007; Ogino et al., 2012). Silva et al. (2018) have employed LCA to quantify the environmental impacts generated in the production of PF and PBM by rendering poultry by-products. However, this study had limitations, since mass allocation was adopted to deal with the environmental impacts generated in the poultry production, which leads to a very high attribution of the environmental impacts of the poultry production chain to the production of PF and PBM. Regarding hydrolyzed feather meal, an interesting protein source also obtained from rendering poultry by-products, to our best knowledge, there are no LCA studies.

This study presents an attributional LCA of poultry fat, poultry by-product meal and hydrolyzed feather meal produced from rendering poultry by-products in two different rendering units in Portugal, which are representative of similar industrial processes in Europe. The rendering processes evaluated followed standardized methods (European Union, 2002) in order to comply with environmental quality and disease control (Meeker & Hamilton, 2006). However, system technologies can vary among rendering units and the raw material composition influences the temperature and the duration of the process applied, which are critical to assure the quality of the final product (Meeker & Hamilton, 2006). This study aims to assess the environmental impacts of PF, PBM and HF production chains analyzing the following impact categories: global warming (GW), abiotic depletion (AD), acidification (AC) and eutrophication (EUT). In addition, PF, PBM and HF have been shown to be able to replace fish oil and fishmeal in aquafeeds (Campos et al., 2017, 2018, 2019), so LCA results of the different ingredients are compared with those of fishmeal and fish oil. Potential strategies to mitigate the impacts of these production chains were identified.

Materials and methods

Goal and scope definition

A life cycle model and inventory were implemented to the production of poultry fat (PF), poultry by-product meal (PBM) and hydrolyzed feather meal (HF) from rendering poultry by-products, following ISO (2006).

The functional unit selected for PF, PBM or HF production was 1 ton of ingredient delivered to the fish feed producer gate (the final consumer of these ingredients).

The impacts were calculated using the CML (Guinée, 2002) Life Cycle impact assessment (LCIA) method for the following categories: abiotic depletion (AD), acidification (AC), eutrophication (EUT) and global warming (GW). These categories were selected for being described in the literature as the most relevant categories in aquafeeds (Papatryphon et al., 2004).

Life cycle model and inventory

Figure 18 shows the system boundaries for the life cycle model ("cradle to gate") implemented to PF, PBM and HF. The system boundaries for PF, PBM, and HF production include the poultry production process, the slaughter process, the transportation of the by-products from the slaughterhouses to the respective rendering plant, the rendering process and the final transportation from the plant to the feed producer, Sorgal S.A..

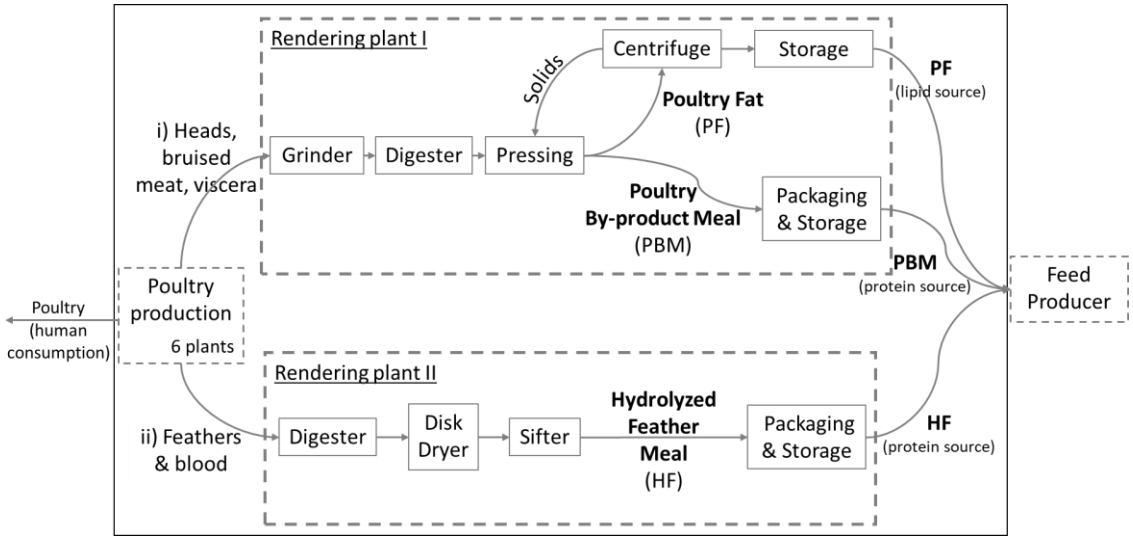

Figure 18. System boundaries of the life cycle assessment of hydrolyzed feather meal (top) and poultry fat and poultry by-product meal (bottom). Dashed boxes represent different locations.

The production of poultry for human consumption generates large amounts of by-products, which can be separated in two different groups: i) heads, bruised meat and viscera,

and ii) feathers and in some cases blood. These two groups are then processed separately into either poultry fat and poultry by-product meal (rendering plant I) or into hydrolyzed feather meal (rendering plant II), respectively, which may be used as ingredients for animal feeds. In 2015 and 2016, the two rendering plants processed about 16 000 and 8 500 tons of poultry by-products (for plant I and II, respectively), generating 1817, 2814 and 2341 tons of PF, PMB and HF, respectively, in 2015, and 1856, 2950 and 2463 tons of the same ingredients in 2016.

In the rendering plant I, the by-products are initially ground to crush larger pieces of meat and bones into smaller pieces and facilitate the digestion process. The resulting product goes into a digester, where it is steam cooked at 101ºC during a minimum of 110 minutes (Method 7, Regulation (EC) No 1774/2002 of the European Parliament) before being pressed to separate the fat from the protein fraction. The resulting fat (PF) goes to a centrifuge (to remove solid particles that return to the pressing phase) before being stored and transported to the feed producer (Sorgal S.A.). The meal that is separated from the fat in the pressing phase (PBM) is packed and stored in big-bags before final transportation.

In the rendering plant II, the raw material (feathers and blood) goes directly into the digester where it undergoes a steam and pressure hydrolysis (133ºC, 3 atm, 20 uninterrupted minutes, according to Method 1, Regulation (EC) No 1774/2002 of the European Parliament). The resulting product is dried in a disk dryer to reduce moisture (below 10%, wet basis), and afterwards sifted to eliminate large particles (> 7 mm). The resulting meal (HF) is packed and stored before being transported to its final destination.

Table 39 shows the main inventory for the two rendering processes, collected from two plants (production data for 2015 and 2016) of the group Soja de Portugal S.A.. Both light fuel oil and wood pellets were used as heat source for the boilers since, due to price variability, the rendering plants alternate between these two fuels. The rendering plant I also consumes natural gas in a cogeneration unit, which supplies process heat to the plant (accounting for 18% of the heat used in the rendering plant). The input of natural gas associated with process heat for the rendering plant I (Table 39) was calculated based on exergetic allocation (Frischknecht, 2000). Emissions for the heat production from light fuel oil, wood pellets and natural gas, as well as the life cycle model of the poultry production and slaughter were calculated based on existing processes in the Ecoinvent database. The impacts for the electricity from the grid were calculated based on the Portuguese electricity mix (Garcia et al., 2014; Marques et al., 2018). The impacts of the infrastructure, capital equipment, and maintenance were assumed out of the boundaries of this study, as they are not significant and usually not considered (Pelletier & Tyedmers, 2007b; Samuel-Fitwi et al., 2013; Silva et al., 2018).

Table 39. Inventory for feather meal and poultry fat production.

	Unit	Production system	
		PF + PBM Rendering plant 1	HF Rendering plant 2
Inputs			
Feathers & blood	t	-	3.574
Poultry by-products	t	8.751	-
Electricity	kWh	451	167
Fuel oil	MJ	7144	1926
Wood pellets	MJ	14778	7671
Natural gas*	MJ	2983	-
Big-bags (polypropylene)	kg	4.02**	2.56
Outputs			
Feather meal (HF)	t	-	1.000
Poultry fat (PF)	t	1.000	-
Poultry by-product meal (PBM)	t	1.569	-
Wastewater	m^3	6.2	2.6

* used in a cogeneration unit;
**big-bags are only used to store PBM.

Poultry fat and poultry by-product meal

The rendering plant I results in different final products: PF, a lipid source ingredient with < 0.15% impurities and < 1% moisture content; and PBM, a protein source ingredient with > 69% protein. Per 8.751 t of poultry by-products input, 1 t of PF and 1.569 t of PBM are produced together (Table 39). Average market prices (PF – 766 $/t, PBM – 824 $/t (Commoprices, 2018; Hammersmith, 2018)) were used to calculate the economic allocation factors (PF – 37%, PBM – 63%). Even though there was some price variability, the price of the two co-products varied in a similar way (PBM ranged from 1.0 to 1.2 times the value of PF).

Hydrolyzed feather meal

Rendering plant II produces HF, a meal that can be used as protein source in animal feeds (> 80% crude protein). To produce 1 t of feather meal, 3.574 t of feathers and blood are required (Table 39).

Multifunctionality

The poultry production system is multifunctional, with heads, bruised meat and viscera, as well as feathers and blood being produced alongside with poultry for human consumption. Per 1 ton of chicken processed, 0.69 t of chicken meat are sold for human consumption, together with 0.22 t of poultry by-products (heads, bruised meat, viscera) and 0.09 t of feathers and blood.

Since the poultry by-products represent a very small percentage (4%) of the economic value of the primary product of the poultry production system (poultry meat for human consumption), economic allocation was used as the preferred approach to calculate the life cycle impacts of the various co-products. The rendering plant I is also multifunctional, as it produces 1.57 t of PBM per 1t PF produced (Figure 18; Table 39). A sensitivity analysis is shown ahead in "Sensitivity analysis to the allocation approach" to assess the influence of adopting mass allocation instead of economic allocation. In addition, since in some cases these by-products could be discarded and not have any economic value, this sensitivity analysis also shows the influence of assuming the by-products as waste (with no environmental impacts from the poultry production phase). Table 40 shows the allocation factors used for a) poultry production and b) PF and PBM production.

Table 40. Allocation factors for a) poultry production, and b) PF and PBM production.

a)	Poultry*	By-products i)	By-products ii)
Mass allocation (%)	69	22	9
Economic allocation (%)	99.1	0.6	0.3

b)	PF	PBM
Mass allocation (%)	38.9	61.1
Economic allocation (%)	37.2	62.8

*for human consumption;
i) heads, bruised meat, viscera;
ii) feathers and blood;
Market values assumed - poultry meat for human consumption: 2000 €/t, by-products i) 40 €/t; by-products ii) 40 €/t; PF: 766 $/t; PBM: 824 $/t.

Transport

Each rendering plant was supplied with the respective poultry by-products by six different slaughterhouses. A weighted average of the amounts transported and the distances travelled was used to calculate the impacts associated with this transport. Besides being supplied by 5 other slaughterhouses, each rendering plant has a slaughterhouse *in situ*. For this reason, part of the raw materials (16 and 23% in the rendering plant I and II, respectively) does not have transportation impacts associated, since internal transport is mechanically done and accounted for in the energy consumption of each rendering plant.

Results and discussion

The following two sections (*"Poultry fat and poultry by-product meal production"* and *"Hydrolyzed feather meal production"*) present the life cycle impact assessments (LCIA) of PF, PBM and HF (based on economic allocation for the poultry production and for the PF and PBM production, and exergy for the heat from cogeneration). The results obtained in these two sections are compared with the LCA of fish oil and fishmeal (obtained in the literature) in the section *"Rendered poultry by-products vs fishmeal and fish oil"*. The section *"Sensitivity analysis to the allocation approach"* presents a sensitivity analysis to allocation type and *"Sensitivity analysis to heat generation"* presents a sensitivity analysis to the fuel used to generate heat in the rendering process.

Poultry fat and poultry by-product meal production

The life cycle impacts calculated for PF and PBM (using economic allocation) are represented in Figure 19. Since these two ingredients are co-products from the same production system, the impacts associated to each of them are similar, and the differences between them are due to the allocation method used and to the use of disposable bags to store PBM, but not PF.

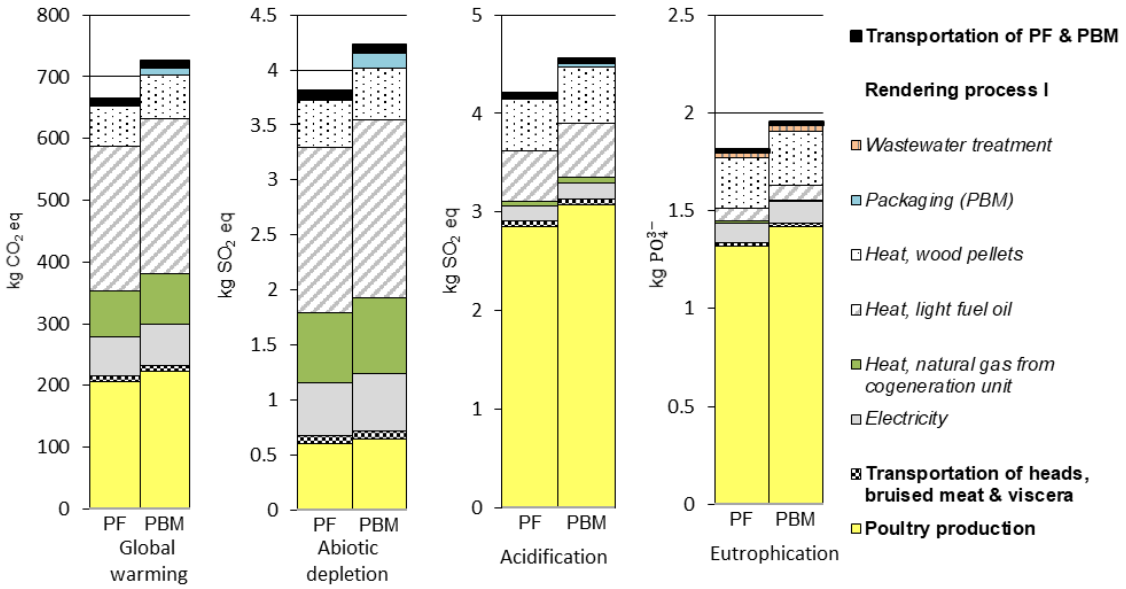

Figure 19. Life cycle impacts of poultry fat (PF) and poultry by-product meal (PBM; 1 t, economic allocation).

The rendering process is the phase with more impacts in terms of global warming (GW, 66% of the total) and abiotic depletion (AD, 80%), but for the acidification (AC) and eutrophication (EU), the major contributor is the raw material (poultry by-products) accounting for 68 and 73% of the impacts of those categories, respectively. Furthermore, since this process also leads to the generation of poultry by-product meal and the impacts were allocated

between the two co-products, the results obtained for the impacts associated to the production of poultry fat are lower than the ones obtained for feather meal production and by comparison the impacts of the other processes seem larger. The transportation of the by-products from the slaughterhouses to the rendering plant and the transportation of the final products (PF and PBM) to Sorgal S.A. (feed producer) had low impacts in all categories (< 5%).

The impacts calculated in the present study for PF and PBM are much lower than the ones previously reported by Silva et al. (2018) for the same ingredients and are also lower than the ones reported for PBM by Pelletier and Tyedmers (2007a). This discrepancy was expected, since the above-mentioned studies used mass allocation over economic, which, in this case, and as further discussed in the next section, gives much more weight to the environmental impacts of the poultry production phase onto the poultry by-products. However, since poultry meat for human consumption and its by-products have completely different economic values, we consider that economic allocation should be the best option in this case. On the other hand, even with economic allocation of the poultry production impacts to its by-products, the poultry production phase remains a major contributor for all the impact categories, agreeing with results shown in the previous studies (Pelletier & Tyedmers, 2007a; Silva et al., 2018). The high environmental impact of the poultry production is related to poultry feed consumption, as it mainly includes crop ingredients and their production and processing have high associated impacts. Furthermore, PBM and PF can be used in animal feeds as a replacement for fishmeal and fish oil, respectively, and these ingredients have higher impacts than PBM and PF for all the categories analyzed in this study (Pelletier & Tyedmers, 2007a; Silva et al., 2018).

Hydrolyzed feather meal production

The impacts of feather meal production (economic allocation) are shown in Figure 20. The rendering process is the phase with more impacts in terms of GW and AD (56 and 72% of the total impacts, respectively), but for AC and EUT, the major contributor is the raw material (feathers and blood), being responsible for 69 and 72% of the total impacts of those categories, respectively. The transport processes of the feathers and blood from the slaughterhouses to the rendering plant and transportation of the final product (HF) to the feed producer, Sorgal S.A., had very low impacts, accounting for less than 10% of the total impacts generated in all categories.

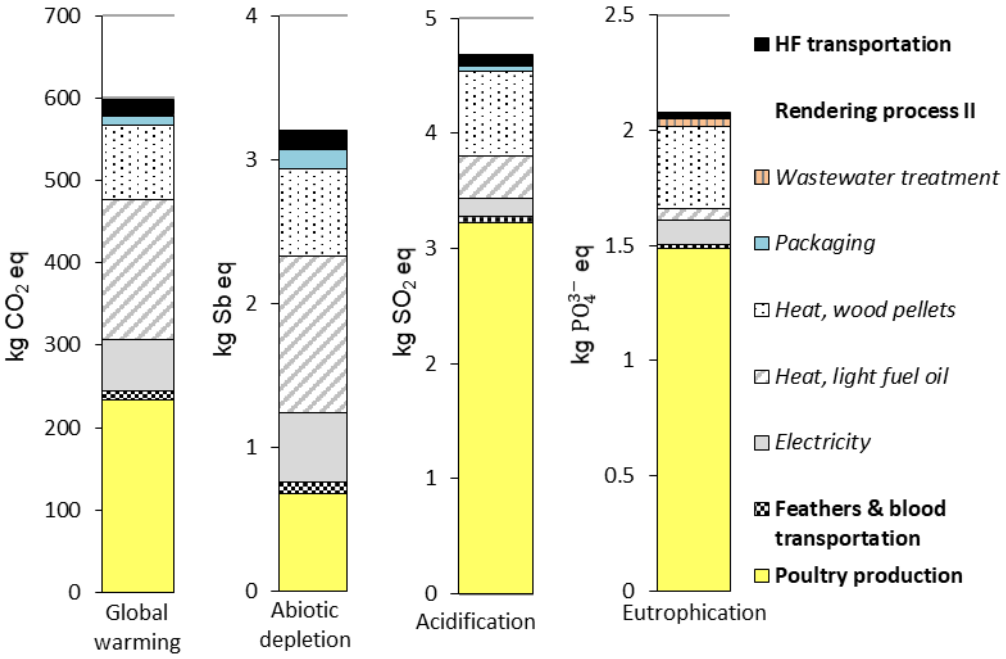

Figure 20. Life cycle impacts of hydrolyzed feather meal (HF; 1 t, economic allocation).

HF can be used as a replacement for fishmeal in animal feeds. Fishmeal has been reported to have higher impacts than the ones reported for HF in all impact categories analyzed in the present study (Pelletier & Tyedmers, 2007; Silva *et al.*, 2018). The high contribution of the poultry production phase to the total impacts of the rendered by-product is consistent with previous reports on the production of poultry by-product meal (Pelletier & Tyedmers, 2007a; Silva et al., 2018). The high environmental impact of the poultry production is related to poultry feed consumption, as it includes mainly crop ingredients and their production and processing have high associated impacts.

Rendered poultry by-products vs fishmeal and fish oil

As previously mentioned, PF has been shown to be able to replace fish oil (FO) in aquafeeds (Campos et al., 2019) while PBM and HF can be substitutes for fishmeal (FM; Campos et al., 2017, 2018). This section compares the results presently obtained for GW, AC and EUT in PF production and in PBM and HF production with literature data on the same impact categories for FO (Figure 21) and for FM production (Figure 22), respectively.

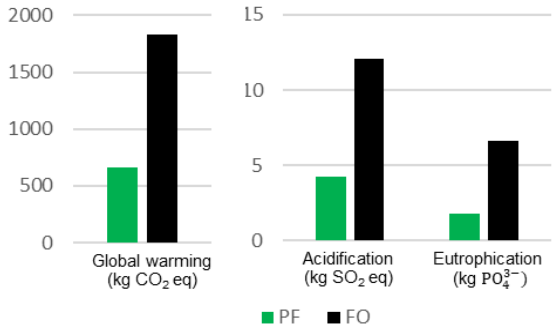

Figure 21. Life cycle impacts of PF and FO (1 t; Pelletier & Tyedmers (2007)).

The impacts of PF for GW, AC and EUT were 666 kg CO_2, 4.2 kg SO_2 and 1.8 kg PO_4^{3-}, while the impacts of FO in the same categories were 1830-2190 kg CO_2, 8.9-12.1 kg SO_2 and 1.7-6.7 kg PO_4^{3-} (Pelletier & Tyedmers, 2007; Silva *et al.*, 2018). For PF and PBM production, the impacts on these categories were 726 and 597 kg CO_2, 4.6 and 4.7 kg SO_2, 2.0 and 2.1 kg PO_4^{3-}, while the impacts reported for FM on the same categories were 1050-1310 kg CO_2, 6.4-6.8 kg SO_2 and 1.2-3.6 kg PO_4^{3-} (Pelletier & Tyedmers, 2007; Silva *et al.*, 2018).

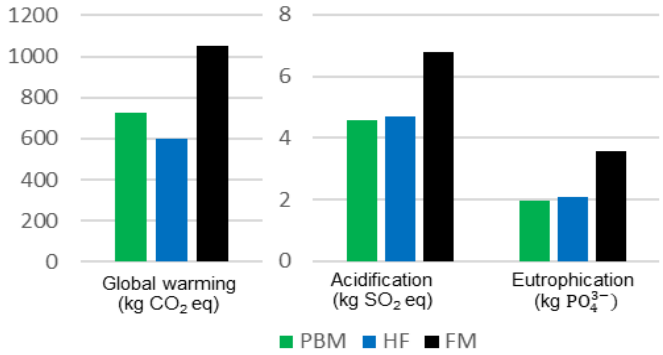

Figure 22. Life cycle impacts of PBM, HF and FM (1 t; Pelletier & Tyedmers (2007)).

Overall, the rendered by-products have lower environmental impacts than FM and FO, indicating that using them as replacements could substantially reduce the environmental impacts of aquafeeds. However, as discussed in the next section, the allocation method applied can have a huge impact in the results obtained. In the studies conducted by Pelletier & Tyedmers (2007) and by Silva et al. (2018), mass allocation was used instead of economic, resulting in a higher attribution of impacts to the poultry by-products and ultimately resulted in higher calculated impacts for the rendered poultry by-products than for FM and FO.

Sensitivity analysis to the allocation approach

The environmental impacts of PF, PBM and HF production, using different allocation approaches (Table 40), are represented in Figure 23. Using economic allocation results in impacts 144, 145 and 164% higher than if considering no impacts from the poultry production industry, for PF, PBM and HF, respectively, for GW (and 119, 118 and 127% higher for AD; 308, 303 and 320% for AC; 366, 365 and 354% for EUT).

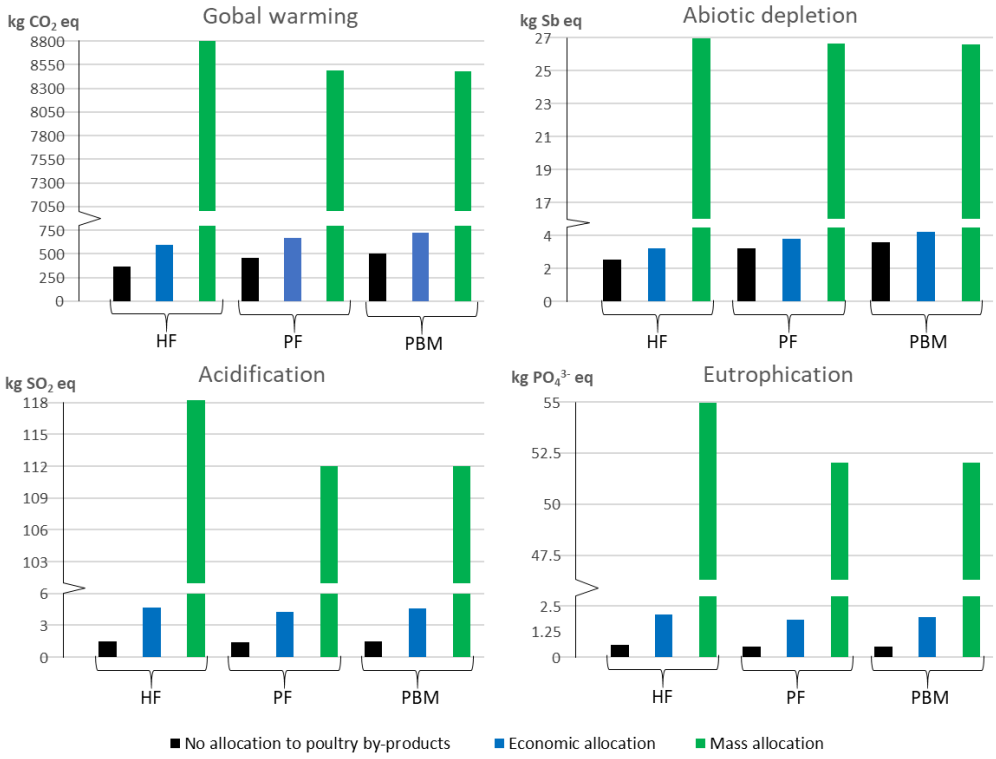

Figure 23. Life cycle impacts of HF, PF and PBM (1 t, at feed producer gate), comparison of different allocation approaches.

However, the use of mass allocation produces extremely high values for all impact categories, when compared to considering no impacts from the poultry production, with a 7 to 11-fold increase in the values obtained for abiotic depletion, 17 to 24-fold increased global warming values, 74 to 82-fold increase in the values for acidification and 93 to 104-fold increase in the impacts calculated for eutrophication. Since the poultry by-products have a much lower price than the main product of the poultry production system (Table 40), it would be more reasonable to allocate the impacts of that system in terms of economic flows than in terms of mass flows. Therefore, in this case mass allocation does not seem to be appropriate and economic allocation was chosen instead to calculate the impacts of these production systems further detailed in this study.

Sensitivity analysis to heat generation

Since the studied production chains are used to valorize poultry by-products, recommendations cannot be made in order to reduce the impacts of the poultry production itself (which is one of the main contributors to the impacts generated). However, since for both production chains the other main contributor to the impact generation is the rendering process, some alterations could be made on the rendering plant in order to reduce the environmental impacts associated with this phase. The impacts in this process are mainly generated in the use of electricity, heat from light fuel oil, heat from wood pellets and heat from natural gas used in the cogeneration unit on the rendering plant I. Both rendering plants use either light fuel oil or wood pellets throughout the year according to market prices. Since the combustion of these two materials have very different impacts, a simulation was made to see the differences between using their current mix, only light fuel oil or only wood pellets as heat source. The results of these simulations are shown in Figure 24.

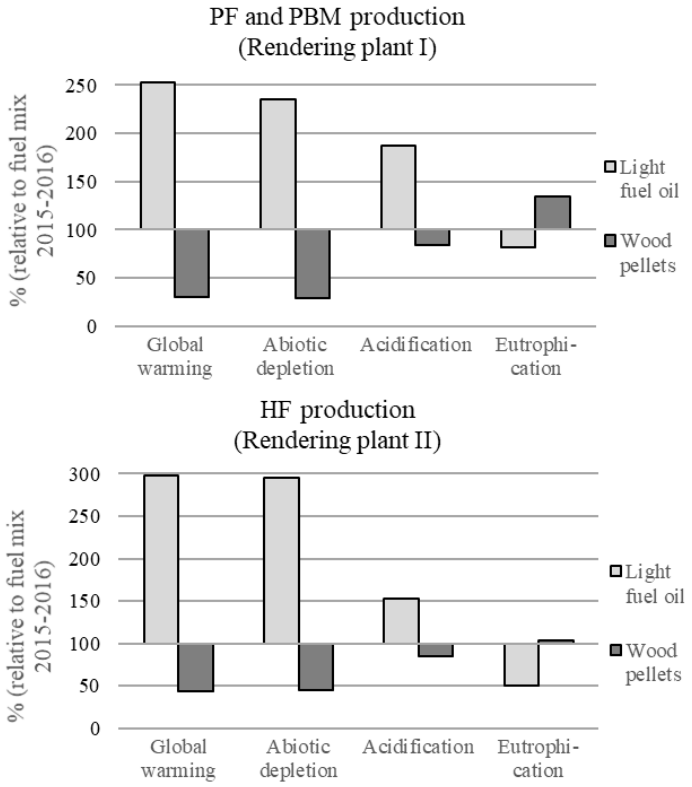

Figure 24. Impacts calculated for poultry fat (PF), poultry by-product meal (PBM) and hydrolyzed feather meal (HF) production (rendering phase) using different fuel alternatives.

In both rendering plants, using only wood pellets as a heat source would reduce the impacts for GW, AD and AC, while slightly increasing the impacts for EUT compared to the mix of fuels used in 2015 and 2016. On the other hand, AD, AC and GW would have huge increases if only light fuel oil was used. This is mainly because these rendering plants already use mostly wood pellets as energy source to produce heat in the rendering process

(particularly in the rendering plant II). However, if only pellets would be used as fuel for heat production, the impacts of this phase could still further decrease substantially. The results obtained in this scenario comparison are consistent with what was previously suggested by Ziegler et al. (2016). These authors suggest that the energy source used during the rendering phase of animal feedstuffs has a big impact on the overall environmental impacts and that the use of renewable energy sources should be privileged over the use of fossil fuels (Ziegler et al., 2016).

Conclusions

The results obtained in the present study show that the poultry production phase is the main responsible for the acidification and eutrophication potential factors in the poultry fat and poultry by-product meal production system and in the feather meal production system. On the other hand, global warming and abiotic depletion factors are mainly influenced by the rendering process of the by-products, in which the main responsible is the fuel mix used in the boilers. A sensitivity analysis showed that a scenario where wood pellets would be exclusively used as heat source in the boilers could reduce all the impact categories considered, except eutrophication. A sensitivity analysis on the allocation method applied to the poultry by-products was also conducted and showed that the impacts of the life cycles analyzed greatly depend on the allocation method, being much higher for all impact categories when mass allocation is used. Overall, the production of PF, PBM and HF from poultry by-products has relatively low impacts for the categories analyzed and could therefore be used as environmentally sustainable feedstuffs in animal feeds. The low impact of such ingredients compared with fishmeal and fish oil indicates that using them as alternative protein and lipid sources would substantially decrease the environmental impacts of animal feed production in the impact categories considered.

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Chapter VIII

General discussion, conclusions and final considerations

While the future availability of fishmeal (FM) and fish oil (FO) to incorporate in fish feeds is at risk, several ingredients arise as possible alternatives. The selection of alternative ingredients must obey several conditions, being not only able to support a good fish growth and final flesh quality, but also enhance the environmental and economic sustainability of aquaculture. However, most of the studies conducted to find alternative protein and lipid sources have focused on vegetable ingredients, which compete with other feed production sectors, and also with direct human consumption. Furthermore, these vegetable ingredients may have to travel far from where they are produced to the place where they are used (i.e. soybean), and therefore have high environmental impacts associated with their transportation, making them in turn environmentally unsustainable. Selecting largely available and nutritious locally produced ingredients that could replace FM and FO would contribute to the sustainable growth of the sector and would also decrease those impacts associated with transportation. This study focused on the replacement of FM and FO with agro-food by-products in diets for European sea bass juveniles. The nutritional quality and nutrient utilization of several agro-food by-products were firstly assessed. The growth performance and flesh quality of fish fed diets with FM or FO replacement was evaluated. Finally, the environmental impacts of those replacements were determined by conducting a Life Cycle Assessment (LCA) of the by-products selected to replace FM or FO.

Fishmeal replacement in European seabass

The first step to evaluate the potential of a certain ingredient is to analyze not only its chemical composition but also its digestibility, which reflects the capability of an animal to use its nutrients. Since the apparent digestibility coefficient (ADC) varies from species to species (due to physiological differences) it is important to ascertain the digestibility of a specific ingredient on the species which will ultimately consume it (NRC, 2011). The classical approach used to assess the nutrient digestibility of a feedstuff consists in comparing a reference diet (with an inert digestibility marker such as chromium oxide) with experimental diets that replace 30% of that diet with the feedstuff to test (NRC, 2011). The relative ADC of each experimental diet and the reference diet can then provide an estimation of the ADC of each tested feedstuff. In the first part of this thesis (Chapter II), several agro-food by-products (wheat germ, okara meal, poultry by-product meal, steam hydrolyzed feather meal, enzymatically hydrolyzed feather meal, β-lactoglobulin and large peptides (>3kDa) obtained from brewer's yeast and from processing fish by-products) were evaluated in European seabass. The results obtained in this thesis show that **hydrolyzed feather meal (steam or enzymatically hydrolyzed), poultry by-product meal, wheat germ, peptides from fish by-products and β-lactoglobulin were all well digested**, resulting in **protein digestibility values above 84%**

(Table 41). The essential amino acids' ADC followed a trend similar to the protein ADC, ranging from 84 to 98% in these ingredients and from 73 to 76% in okara and yeast peptides.

Table 41. Protein and energy content and ADCs in European seabass agro-food by-products studied in the present thesis. Values previously reported for the same ingredients in seabass are given within brackets.

Ingredient	Protein		Energy	
	% DM	ADC (%)	kJ/g DM	ADC (%)
Wheat germ	28[1]	97	21	89
Okara	27[1]	70	21	64
Poultry by-product meal	72[1] (60-62)[2,3]	93 (86-97)	22 (21)	90 (97)
Steam hydrolyzed feather meal (HF)	86[1] (77)[2]	85 (67)	24 (24)	80 (58)
Enzymatically HF	86[1] (82)[2]	88 (68)	24 (23)	85 (61)
β-lactoglobulin	79[1]	99	24	95
Yeast peptides (>3 kDa)	39[1] (48)[3]*	78 (89)	20	61 (85)
Fish by-product peptides (> 3kDa)	86[1]	97	21	88

1 - Chapter II; 2 - Davies et al. (2009); 3 - Tulli and Tibaldi (2001);
* the values presented within brackets for this ingredient correspond to whole brewers' yeast.

Previous results in the literature reported protein ADC values of 90 – 96% for FM (Davies et al., 2009; Gomes da Silva & Oliva-Teles, 1998). The present results show that most ingredients were equally well digested and in sime cases, like wheat germ, the peptides from fish by-products and β-lactoglobulin, even exceeded that value range. This suggests that such agro-food by-products would be able to replace dietary fishmeal without impairing diet digestibility. Furthermore, all these ingredients are produced locally, having the potential to reduce the need for imported feedstuffs and consequently the environmental impacts associated with feedstuff transportation, as they can be produced closer to the end user.

However, other characteristics must be considered when selecting FM replacers for feed formulation, namely the ingredients' nutritional quality (including protein content and amino acid profile), availability and price of the processed by-products. The protein content of some of the above-mentioned feedstuffs, for instance wheat germ, was very low, indicating that even though this ingredient has a great digestibility in seabass, a direct replacement could never be feasible since it has only about a third of the FM protein content. Nonetheless, since this ingredient has been shown to have anti-hypertensive properties and other nutritional benefits (Brandolini & Hidalgo, 2012), it could be an interesting ingredient to use as a supplement or a partial FM replacer (Reis et al., 2019). On the other hand, β-lactoglobulin and the large peptides obtained from processing fish by-products are protein-rich ingredients with outstanding digestibility results. Nevertheless, their current production volume is not enough to assure its utilization as an aquafeed feedstuff and further technological developments would be required to assure a cheaper and larger production. However, it should be highlighted that the technological processes used to obtain these larger peptides also produce peptides with lower molecular weight that have bioactive properties applicable to human health and nutrition.

Contrarily, the feedstuffs that were obtained from poultry by-products (poultry by-product meal and feather meal) are generated in large amounts. Poultry by-product meal is a protein-rich ingredient and proved to have a high protein digestibility in European seabass, being a very interesting protein source (in fact, it is already used in some seabass commercial diets). Different hydrolysis methods were applied to the feathers but resulted in similar ADC values in protein and in most individual amino acids, with the exception of methionine and serine that were better digested in the enzymatically hydrolyzed feather meal (94 *vs* 100% and 61 *vs* 74%, respectively). This indicates that these amino acids become less available when the steam/pressure treatment is applied, but also suggests that the enzymatic method does not seem to be advantageous compared to the steam hydrolysis method, as it was about 50% more expensive.

Considering the ADC values of the analyzed feedstuffs, mainly for protein and essential amino acids, alongside with their availability and price, **steam hydrolyzed feather meal (HF) was selected as a FM replacement in juvenile seabass diets.**

In Chapter III, the impact of including 5, 7.5 and 12.5% of HF in diets for juvenile seabass, replacing 28, 55 and 76% FM, was evaluated in a growth trial of 18 weeks. Results showed that the dietary inclusion of HF up to 12.5% (76% FM replacement) **did not affect diet palatability**, generating **similar feed intake**, **feed conversion ratio** and **protein efficiency ratio** among dietary treatments. It is important to highlight that the HF used contains not only feathers but is rather a blend of poultry feather and blood from the slaughtering process (the mix contained about 25% blood), which might help explain such high feed acceptability by the fish. It is also imperative to point out the importance of the dietary supplementation with L-Lysine and DL-Methionine in order to fulfill the requirements of European seabass when FM was replaced by HF, since this ingredient is deficient in those amino acids. Most of the studies previously reported on literature using HF in fish did not balance the amino acid profile of the diets, which could explain the growth impairment obtained with lower HF levels (40% FM replacement) than those used in this study (Yu, 2008).

When FM was replaced at the highest level (76%), lower protein and energy ADCs were observed (90.3 and 82.2%, respectively, *vs* 91.5 and 85.0% in the FM diet), but **energy and nutrient gain remained similar** among dietary treatments. This ultimately led to **similar final body weight**, and final whole-body composition among experimental diets. Furthermore, the **dietary HF inclusion improved phosphorus ADC**, **significantly decreasing fecal P losses into the environment**. However, the lower protein ADC obtained with the inclusion of 12.5% HF was associated with higher nitrogen fecal losses. This indicates that while the inclusion of HF can decrease P emissions into the environment, as previously suggested by

Sugiura et al. (2000) in rainbow trout, high levels of dietary inclusion (> 7.5%) may also result in a higher nitrogen discharge into the water, which could result in the pollution of aquaculture effluents if not properly treated.

The **muscle EPA and DHA levels remained unaffected by the FM replacement by HF** even when the diet with highest replacement level was used. In fact, all fish had **levels above those recommended for human consumption** to decrease the risk of cardiovascular diseases (0.25 – 0.5 g per 100 g portion of fish; EFSA Panel on Dietetic Products & Allergies, 2010), demonstrating a great nutritional value for consumers' health (Sargent, 1997). In addition, it also must be highlighted that FM replacement up to 76% had no effects on European seabass immune status, supporting previous results in Atlantic salmon after a 44% fishmeal substitution (Bransden et al., 2001) and indicating that this feedstuff does not seem to impair seabass health. Nevertheless, such results have to be analyzed with caution and a challenge trial should be further carried out.

Finally (Chapter VII), and compared to FM, the life cycle assessment of HF showed that this ingredient has **lower impacts in all categories studied** (global warming, abiotic depletion, acidification and eutrophication potential). Impact values **for global warming were almost 50% lower in HF compared to FM**, for instance. The life cycle of poultry by-product meal (PBM, a coproduct of PF that was also considered an interesting FM substitute) was also evaluated. The results obtained were similar to those of HF for most categories, but higher global warming impacts were obtained in the PBM production. However, the life cycle of PBM reported lower impacts than FM for all the categories assessed.

In conclusion, HF is well digested by European seabass and can be **included up to 12.5% in juvenile seabass diets (replacing 76% of the dietary FM) without impairing** either **growth**, **immune status** or **EPA and DHA levels in the muscle**, suggesting that this can be a very good protein source to incorporate in feeds for this species. Moreover, **using this ingredient** as a replacement for FM **decreases protein digestibility, increasing nitrogen emissions into water.** On the other hand, **it improves phosphorus digestion, decreasing its emissions into the water,** and the LCA results show that HF can considerably **decrease the environmental impacts associated with aquafeeds**, also contributing to the circular economy by reintroducing such by-products into the local economy.

Fish oil replacement in European seabass

When considering fat sources in fish diets, similarly to protein sources, the first approach concerns the evaluation of their digestibility, which is an important tool to evaluate the potential of a certain ingredient as a feedstuff. Rendered fats can be sustainable alternatives to FO due to their lower price and wider availability, but their digestibility by European seabass has not been determined before. Nevertheless, the classic approach for ADC studies, where 30% of a reference diet is replaced by a certain ingredient (NRC, 2011), is not feasible for fat sources. This is mainly due to the extremely high fat content of the resulting experimental diets that would impair the extrusion process. The strategy used in this thesis to overcome this obstacle was the formulation of a reference diet with FO as supplemental fat source; then, each experimental diet resulted from the complete replacement of the supplemental FO by each lipid source to test. Even though this does not allow the determination of the ingredient's ADC according to the formula proposed in NRC (2011): ADCing (%) = ADCtest + [(ADCtest − ADCref) × ((0.7 × Dref) / (0.3 × Ding))], the comparison of the nutrients' ADCs among the experimental diets enables an overall evaluation of the effect of the supplemental fat source, since it is the only variable among diets. This strategy has been recently applied by Yılmaz and Eroldoğan (2015) to evaluate vegetable oils (rapeseed and cotton oil) in seabass. In Chapter IV, three different fats (fish oil, poultry fat (PF) or mammal fat (MF)), all obtained from rendering animal by-products were used as supplemental fats in the experimental diets used to evaluate the *in vivo* digestibility as described above. Each diet was tested in quadruplicate using new groups of fish to get four independent replicates per diet, allowing a more robust statistic. ADC values obtained for SFA, MUFA and PUFA in seabass are presented in Table 42 alongside with the presently obtained results for rendered animal fats.

Table 42. Comparison of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid levels (% total fatty acids) and their respective ADC values in diets with different lipid sources in European seabass.

Supplemental lipid source	SFA		MUFA		PUFA	
(% inclusion)	%	ADC (%)	%	ADC (%)	%	ADC (%)
Fish oil (10)[1]	27.0	96.1	39.2	99.4	33.8	98.8
Rapeseed oil (10)[1]	18.2	96.0	55.1	99.2	26.7	97.1
Cottonseed oil (10)[1]	28.7	95.7	23.0	97.3	48.3	96.8
By-product fish oil (13.8)[2]	29.9	91.0	30.2	95.1	35.7	97.6
Poultry fat (13.8)[2]	33.2	91.5	43.3	95.3	21.8	95.6
Mammal fat (13.8)[2]	33.6	85.4	43.0	94.4	21.6	97.0

1 – Yılmaz and Eroldoğan (2015); 2 – Chapter IV.

The results obtained in the digestibility trial showed that lipid and energy ADCs of the various fat sources were generally high (> 86%) without differing significantly among diets. However, even though the ADCs of individual fatty acids were high (78 – 98%), ADC values

varied according to the fat source: compared with the FO diet, diets with **land animal fats** not only **had low levels of EPA and DHA** (provided by the dietary fishmeal) **but also resulted in decreased digestibility of those fatty acids**. The fatty acid profile of the land animal fats (rich in SFA and MUFA) created SFA-rich diets (> 30% of the total fatty acids) with decreased PUFA/SFA ratios, which have been reported to impair emulsion or the formation of micelles (Menoyo et al., 2003) and reduce lipolytic activity (Caballero et al., 2002), respectively, compromising the digestibility of unsaturated fatty acids. Also, since the rearing temperatures of European seabass vary throughout the season and the farm location (5-28 ºC; FAO (2017)), the ADC of land animal fats should be evaluated in a wider temperature range. These animal fats have high amounts of SFA which have lower melting points than PUFA or even MUFA, so they tend to be solid at room temperature. Since fish are poikilothermic, diets richer in SFA usually result in impaired digestibility under colder water temperatures (< 21 ºC), affecting the outcome of using these fats, but that was not the case at higher temperature.

In conclusion, the **high energy and total lipid ADC values of the different rendered land animal fats make them valid energy sources to replace FO in seabass diets.** However, the low levels or absence of EPA and DHA in those fats allied to their decreased digestibility, may compromise the dietary inclusion of animal fat sources at high levels. Furthermore, even though total PUFA were better digested in MF than in PF, the latter is much richer in PUFA than the former, being particularly rich in 18:2*n*-6 and 18:3*n*-3, which are essential fatty acids for fish. Considering these results, **both animal fats were considered able to replace fish oil** and were used in two subsequent growth trials with European seabass juveniles – one trial evaluated **PF as single fish oil replacer** and in the other trial **MF was blended with PF (50:50)** to enable higher PUFA levels **and this mixture (PFMF) was used as fish oil replacer**.

Dietary lipids are mainly used as a source of energy having a high impact on fish metabolism, lipid deposition and tissue lipid composition. Therefore, all of these parameters should be taken into consideration when replacing dietary lipids. In Chapter V, the impacts of replacing 25, 50, 75 and 100% fish oil by PF (25PF, 50PF, 75PF and 100PF) were evaluated in a growth trial of 16 weeks. Soy lecithin was also tested as an emulsifier agent in the diet with 100% PF (100PFL), as it could help surpass digestibility issues associated with such high FO replacement. In Chapter VI, the impacts of replacing 50, 75 and 100% by PFMF (25PFMF, 50PFMF and 100PFMF) were evaluated in a growth trial with the same duration. Since the reference diet used for both trials was the same and the only difference among treatments was the supplemental oil source, the results obtained in Chapter V will be discussed alongside those obtained in Chapter VI.

Feed intake was not affected by any of the diets, indicating **good diet palatability**, and the **feed conversion ratio**, **protein efficiency ratio** and **daily growth index were similar** among experimental diets in both growth trials.

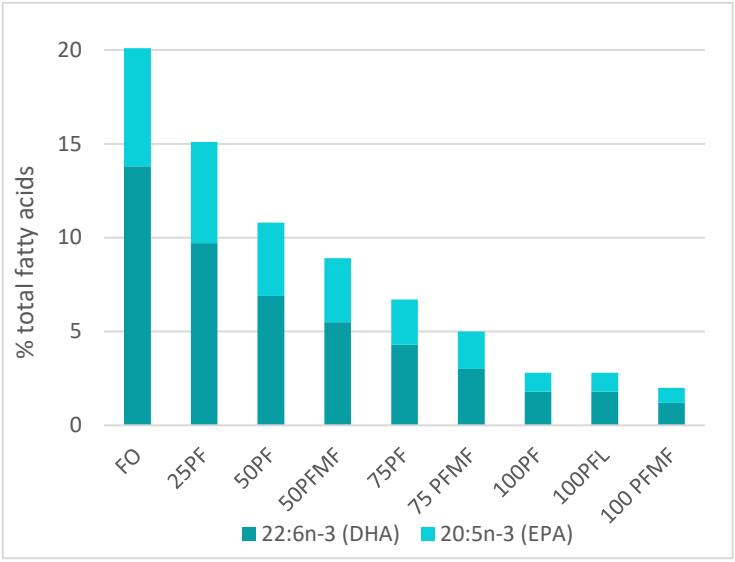

Figure 25. Dietary EPA and DHA levels (% total fatty acids) of the different experimental diets including increasing PF and/or MF (25-50-75-100%) to replace FO.

The increasing FO replacement throughout the experimental diets of the two growth trials let to a progressive decrease in the dietary EPA and DHA levels and a consequent dietary n-3/n6 fatty acids ratio decrease, as shown in Figure 25 and Figure 26.

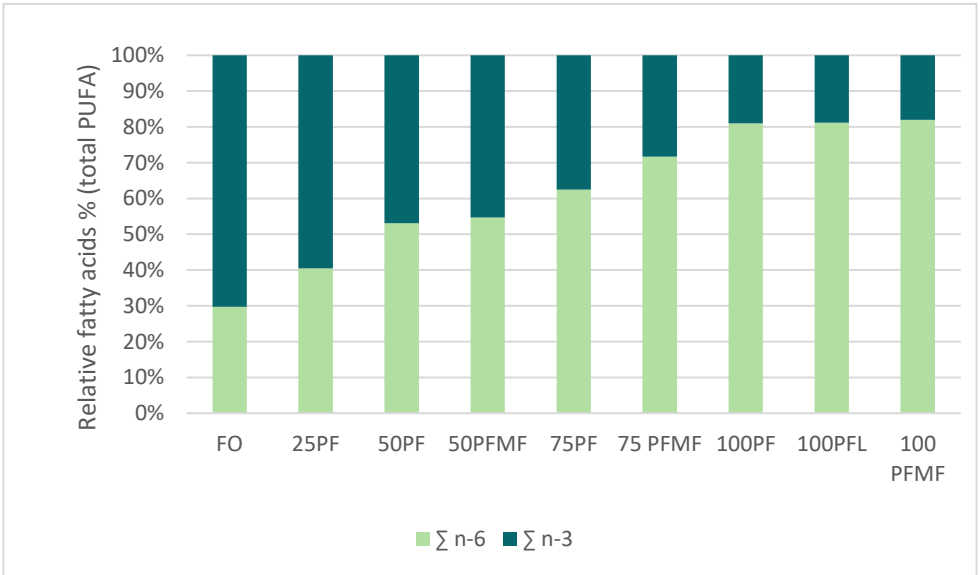

Figure 26. Relative dietary ratios of total n-3 and n-6 PUFA (% total PUFA) of the different experimental diets including increasing PF and/or MF (25-50-75-100%) to replace FO.

Despite the sharp decrease in dietary n-3/n-6 ratio and dietary EPA and DHA levels, the growth performance of seabass did not mirror these dietary alterations. In fact, even though diets with the lowest EPA and DHA levels resulted in the lowest weight gain observed (in 100PFMF), the inverse is not true, as fish fed the diet with highest levels of these fatty acids (FO) did not have the highest weight gain (Figure 27).

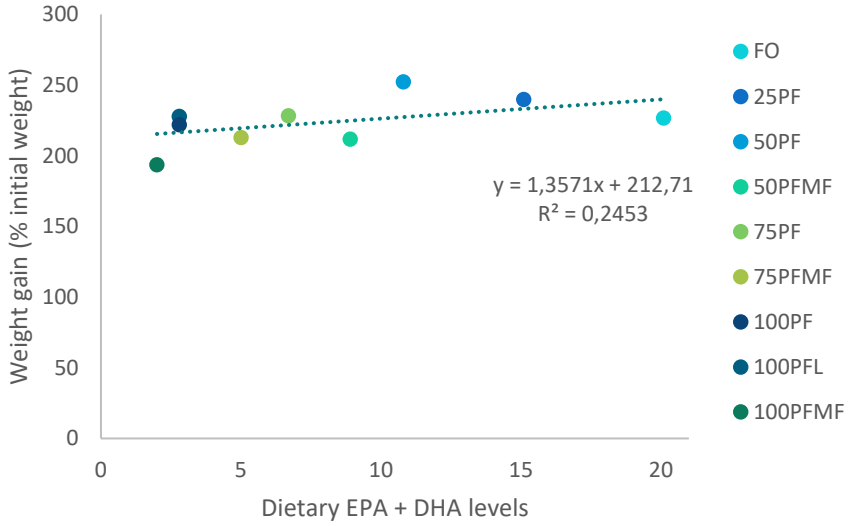

Figure 27. Weight gain of seabass fed the FO diet and the different experimental diets with PF or PFMF as fish oil replacement (% initial body weight) expressed regarding the respective dietary EPA + DHA levels.

The present results show that there is no clear correlation between dietary EPA and DHA levels and weight gain, indicating there are other factors involved in growth regulation when dietary lipids are changed. These may include differences in fatty acid profiles, since PF diets generally provided better results than PFMF, even when their EPA and DHA levels were similar. For instance, PF is richer in 18:2*n*-6 and 18:3*n*-3 than MF and therefore diets with PFMF as fish oil replacement had lower levels of those fatty acids than diets with the same level of replacement using only PF (Figure 28). Since 18:2*n*-6 and 18:3*n*-3 are also essential fatty acids for fish growth, this may also be partly responsible by those differences.

Furthermore, as shown in Figure 28, since MF is richer in saturated fatty acids than PF, diets with PFMF are also richer in those fatty acids, which have been shown to hinder overall digestibility and could be responsible for the decreased growth observed. In fact, while **the inclusion of PF up to 100% did not affect nutrients' digestibility**, **when fish oil was completely replaced by PFMF lipid ADC decreased significantly**.

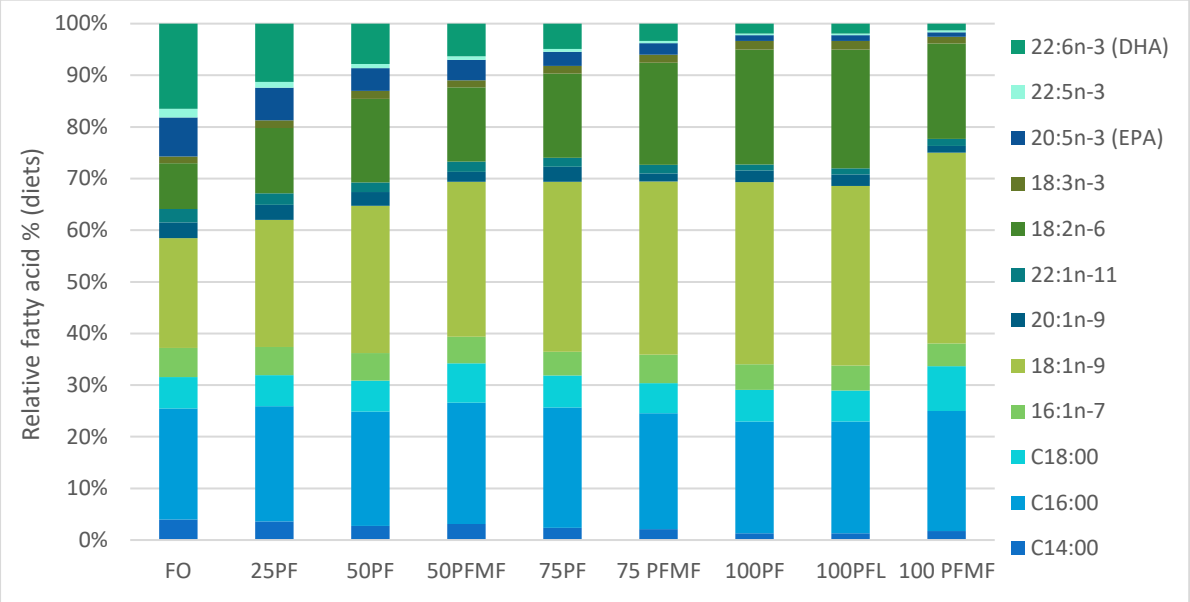

Figure 28. Dietary fatty acid proportions in the experimental diets used to test fish oil replacement.

Final body weight was also only impaired in fish fed the 100PFMF diet, remaining unaffected by the dietary lipid source with all other experimental diets with either PF or PFMF as fish oil replacement. Thus, it seems that **it is possible to replace 75% dietary fish oil with PFMF and totally replace it with PF without impairing European seabass juveniles' feed intake, growth performance and nutrient utilization.** However, it is important to mention that even though supplemental fish oil has been completely replaced by PF, all the essential fatty acids were still provided by the fishmeal included in that diet. Further studies would be required to evaluate the effect of replacing fish oil with PF in diets with lower levels, or even no fishmeal. The whole-body composition remained unaffected by the dietary lipid source. However, the hepatic lipid content was significantly increased when fish oil was completely replaced by either PF or PFMF. However, **including soy lecithin significantly attenuated the increased liver lipid content in fish fed PF-based diets.** The effect of this emulsifier was not evaluated when using PFMF as a fish oil replacement and further studies would be necessary to see if the effects would be similar with that lipid source. The increased hepatic lipid content resulted in increased hepatosomatic indexes which led to and hepatic vacuolation and apparent steatosis, also compromising glycogen deposition. This hepatic lipid accumulation may have been triggered by insufficient dietary phospholipids due to the fish oil replacement, since phospholipids are required for the lipoprotein synthesis. This could explain the attenuation of hepatic fat deposition when soy lecithin was supplemented in 100PFL, an effect previously observed in Arctic char (Olsen et al., 1999).

The fatty acid profile of the tissues analyzed in both trials generally reflected the dietary lipid source, increasing MUFA and decreasing PUFA contents with PF and PFMF inclusion (Figure 29 and Figure 30). This was also true for the muscle EPA and DHA content, and even though there was a preferential deposition of these fatty acids on this tissue, there was a significant reduction of their levels with the decrease of dietary fish oil.

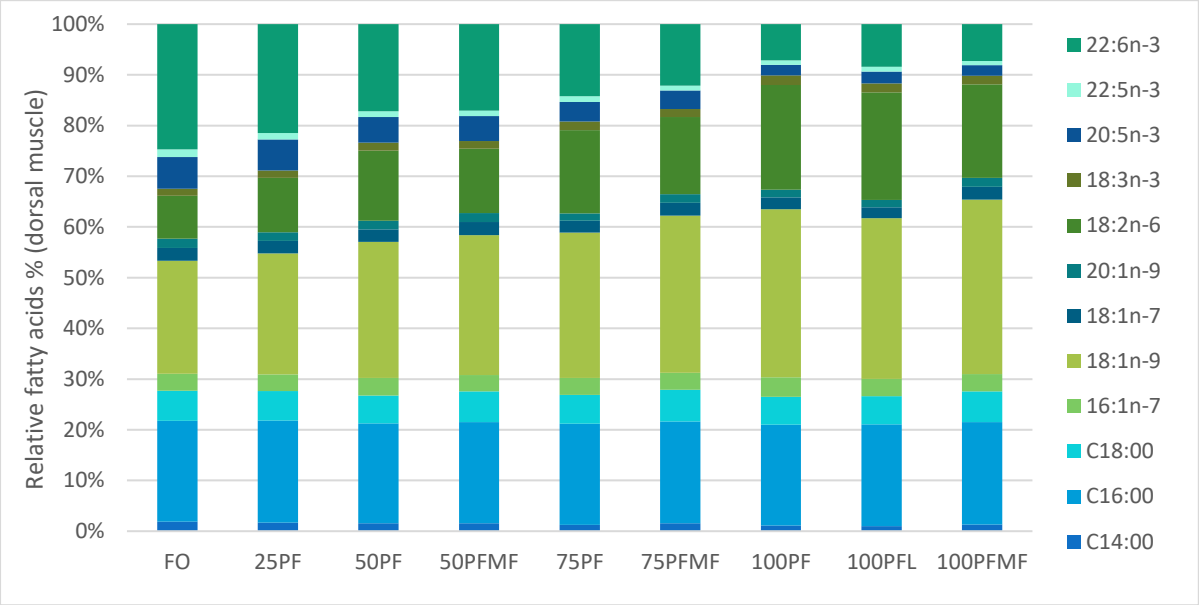

Figure 29. Dietary fatty acid proportions in the dorsal muscle of fish fed the experimental diets including increasing PF and/or MF (25-50-75-100%) to replace FO.

From a consumer's point of view, fish consumption is associated with health-benefit effects. So, the replacement of fat sources in aquafeeds has a major impact on flesh FA profile. But despite these alterations, the dorsal muscle of seabass fed up to 75% PFMF still provided the recommended daily intake levels of EPA + DHA (0.3-0.5 g per 100 g of muscle; EFSA Panel on Dietetic Products & Allergies, 2010). On the other hand, while fish fed PF diets provided the recommended EPA and DHA levels on dorsal muscle only up to 50% replacement, the recommended values were still provided by fish fed the diet with 75% replacement in the ventral muscle, which is also an edible part of fish appreciated by consumers (Testi et al., 2006). However, even considering both dorsal and ventral muscle, EPA and DHA were bellow recommended levels for human consumption for fish fed PF or PFMF-based diets (100PF, 100PFL and 100PFMF). Plus, the replacement of 75% by PF or by PFMF led to fillets with decreased hypocolesterolemic/hypercolesterolemic fatty acids ratios and lower atherogenic (AI), thrombogenic (TI) and peroxidation indexes. Even though the seabass used in these growth trials did not reach commercial size, these results suggest that the resulting **fillets of fish fed 75PF and 75PFMF would** not only **have a longer shelf life** (due to their lower peroxidation index) but also still **provide the recommended levels of EPA and DHA for human consumption.** Moreover, **such diets may also reduce the risk of lipid**

deposition in blood vessels (indicated by the AI and TI) **and hypercholesterolemia,** potentially **improving the consumer's coronary heart health.**

The long-term effects of using PF and PFMF as fish oil replacement warrant further evaluation to determine if these trends would be sustained with fish reaching commercial size. Moreover, such low *n*-3 and high SFA dietary levels should also be tested in pilot trials under variable temperature and salinity conditions, to fully understand their impact on seabass growth performance and health status.

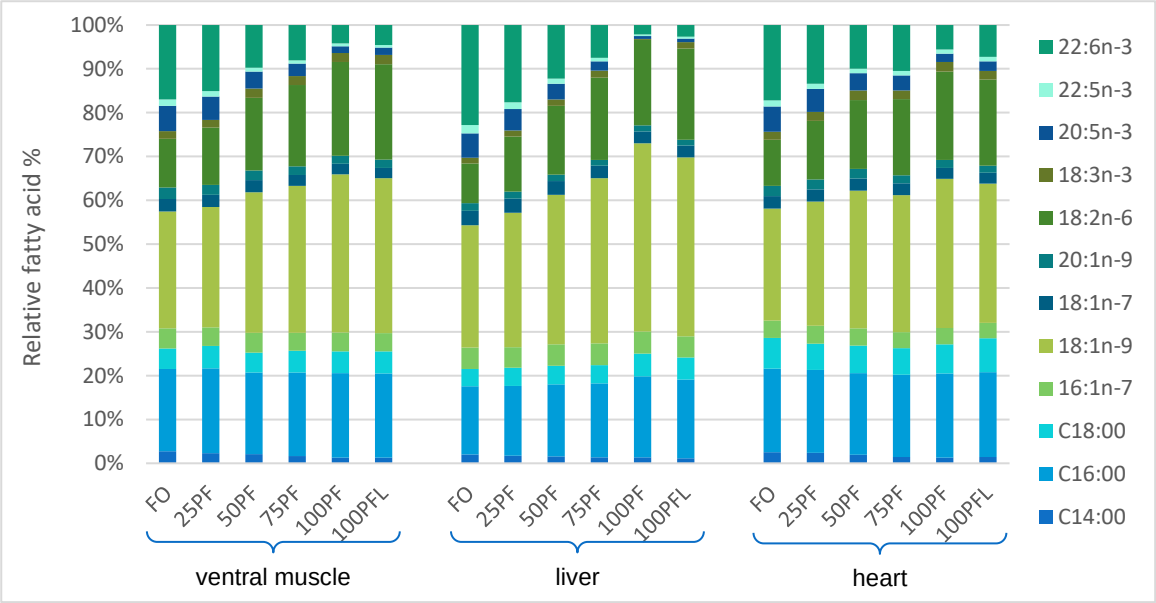

Figure 30. Dietary fatty acid proportions in different tissues of fish fed the experimental diets used to test PF as a fish oil replacement.

The effects of including soy lecithin in the PF-based diet were evident in attenuating the increased liver lipid deposition and the decrease in the heart n-3/n-6 ratio (Figure 30). Since PF has a high melting point (about 28 ºC), high dietary levels of this fat could result in digestibility issues (Bureau et al., 2002), which motivated the inclusion of soy lecithin as an emulsifier. However, the relatively warm water temperature at which this trial was conducted (21 ± 1 ºC) may have helped surpass these digestibility issues, resulting in no major advantages in the use of this additive. As previously mentioned for the digestibility study, seabass can be reared in a wide temperature range. Therefore, the use of PF and PFMF in seabass diets, as well as the inclusion of soy lecithin, should be evaluated in lower water temperatures, since this could have an impact on the effects of using these fats and soy lecithin could be a valuable additive in those conditions.

Finally, since **PF and MF** are obtained by rendering locally produced by-products, their **use in seabass diets would contribute to a circular economy**. The life cycle of PF was analyzed and showed that, when compared with fish oil, PF generates less than half the

environmental impacts in all the categories evaluated (global warming, abiotic depletion, acidification and eutrophication potential), with values almost 3 times lower for global warming. Thus, **using PF as a fish oil replacement in aquafeeds should substantially reduce the environmental impacts associated with this industry**. The LCA of MF was outside the scope of this thesis and therefore was not evaluated. However, in order to evaluate the potential environmental benefits of using this fat as fish oil replacement, the evaluation of its life cycle should also be conducted.

In conclusion, the results presented in Chapter IV, Chapter V and Chapter VI show that PF and MF are well digested by seabass and while the **total replacement of fish oil by PF was possible without impairing feed intake, growth performance and nutrient utilization**, this was only possible **up to 75% with the PF and MF mix** (37.5% PF + 37.5% MF). This indicates that **PF would be a better alternative lipid source than MF in European seabass diets**. Yet, it is important to highlight that even though supplemental fish oil has been completely replaced (or almost completely in the PFMF case), these diets still had about 25% fishmeal, which was able to provide all the essential fatty acids. Despite the alterations of the muscle FA profile, **fillets of seabass fed 75PF and 75PFMF** still **provide the recommended levels of EPA and DHA for human consumption** while also showing a potential for **reducing the risk of lipid deposition in blood vessels and hypercholesterolemia,** possibly **improving the consumer's coronary heart health**. Additionally, **using these by-products as fish oil substitutes will reintroduce them into the local economy, contributing to a circular economy and possibly reducing substantially the environmental impacts associated with the aquaculture industry**.

Main conclusions

The work conducted along this thesis, which was carried out in an industrial setting in partnership with the company Soja de Portugal, allowed for the evaluation of several agro-food by-products as potential fishmeal and fish oil replacements to be used by the aquafeed industry. The results obtained and presented in the previous chapters provide information that enables the feed industry to make more responsible decisions when selecting ingredients for feed formulations, assuring a more sustainable development of the sector. From the results herewith obtained, the following conclusions can be formulated:

- Wheat germ, fish by-product peptides, β-lactoglobulin, poultry by-product meal, steam hydrolyzed feather meal and enzymatically hydrolyzed feather meal are protein sources well digested by European seabass (> 84% protein ADC).

- Replacing up to 76% fishmeal with steam hydrolyzed feather meal did not negatively affect growth performance, nutrient gain, immune status and muscle EPA and DHA levels, and the inclusion of this ingredient in seabass diets improved phosphorus ADC, significantly decreasing its emissions into the environment.

- PF and MF are well digested by seabass, presenting high energy and total lipids ADC compared with FO, but their inclusion as fish oil substitute decreased the dietary EPA and DHA levels and their ADC values.

- Replacing fish oil without impairing feed intake, growth performance and nutrient utilization was possible up to 100% replacement when using PF or up to 75% using the PF and MF mix (37.5% PF + 37.5% MF), indicating that PF would be a better alternative lipid source than MF in seabass diets.

- Up to 75% fish oil replacement by either PF or PFMF, seabass muscle still provides the recommended levels of EPA and DHA for human consumption and shows a potential to improve the consumer's coronary heart health.

- The life cycles of HF and PBM, as well as the life cycle of PF, generate less impacts than the life cycles of fishmeal and fish oil, respectively, in all the categories analyzed, indicating that using such land animal sources as FM or FO replacements would substantially decrease the environmental impacts associated with aquafeeds.

- The use of pellets as heat source in the rendering plants where HF, PF and PBM are produced could decrease the environmental impacts associated with their production.

Future perspectives

The research conducted in this thesis showed that there are multiple agro-food by-products with great potential as protein sources for seabass due to their nutritional quality and high protein/amino acid digestibility. However, new technological procedures should be further developed to assure cheaper and wider production of some of the agro-food by-products studied, since their current volume and production methods make them unsustainable to use in aquafeeds as protein sources.

All the growth trials conducted within the scope of this thesis were performed with juvenile seabass in short periods. Although such short-term trials provide valuable information, it would be extremely important to evaluate the effects of such replacements in the long-term by conducting pilot trials, which would enable a validation of the conclusions herewith drawn for the final seabass fillets in commercial size fish. Furthermore, the use of poultry fat and mammal fat as fish oil replacement should also be studied under variable temperature and salinity conditions. This would enable a better evaluation of their impact on seabass growth performance and health status, providing more reliable and practical information for the feed industry concerning the most appropriate inclusion levels for seabass farming under varying conditions. The effect of soy lecithin should also be tested as an emulsifier when using these fats at lower water temperatures, as these conditions can lead to impaired digestibility when using land animal fats, rich in SFA.

As previously stated, the diets used to test fish oil replacement still had about 25% fishmeal, which provided all the essential fatty acids required by seabass. Further investigation would be required to evaluate the effect of replacing fish oil by land animal fats when dietary fishmeal levels are lower or even none. Furthermore, since the total replacement of fish oil by poultry fat decreased the muscle EPA and DHA levels but still provided good growth performance, it would be interesting to see if a finishing fish oil-rich diet would improve the final fillet quality without compromising the sustainability of the aquafeeds.

Finally, it would be important to evaluate the life cycle of mammal fat, to quantify the potential environmental benefits of using this ingredient as a fish oil replacement. Also, a consequential LCA study should be conducted to further evaluate the potential impacts of replacing FM and FO by land animal by-products. A consequential LCA would evaluate the impacts of performing such replacements in the production chains of each ingredient (as consequence of a change in demand), whereas the LCA performed in this thesis (attributional LCA) allowed only for the characterization and comparison of the environmental impacts of each production system.

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